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SEGREGATION OF ANTIGENS FROM HUMAN LEUKEMIC AND TUMORAL CELLS BY FLUOROCARBON EXTRACTION

I. DETECTION BY A GEL DIFFUSION METHOD

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WITH THE TECHNICAL ASSISTANCE OF L. RENERTS

After fluorocarbon homogenization of human leukemic and tumoral cells, two main fractions were separated, one a ribonucleic acid–rich and the other a protein-rich fraction. Since weak antigenicity had been found previously in the RNA-rich fraction, antigen analysis of the protein moiety was attempted. When tested by a gel diffusion method against both animal antisera prepared with proteins from malignant cells and against antisera prepared with proteins from normal tissues, the tumoral proteins showed antigens common to both normal and malignant fractions and, in addition, antigens specific for the malignant fractions. The normal antigens, but not the malignant ones, could be removed from the whole malignant protein extract by absorption of the latter with animal antinormal gamma globulin. The specific antigens found in solid tumors were different from the ones found in leukemias and, within the leukemias, there were different antigens in acute stem cell and in chronic lymphatic leukemias.

IN PREVIOUS reports[1-5] several interesting biologic properties of a ribose nucleic acid (RNA) fraction obtained by extensive fluorocarbon homogenization of human leukemic and tumoral cells were presented. These included cytopathogenicity for cultures of human amnion cells and leukemogenic and oncogenic activity in leukemia- and tumor-free strains of mice. Rabbit antisera produced with a microsomal fraction from human leukemic cells was shown to

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be cytotoxic for the homologous leukemic cells in vitro. When the RNA fraction was used to immunize animals, weak neutralizing antibodies and precipitins were obtained. On the other hand this fraction displayed a marked hemagglutinating capacity for fresh rabbit erythrocytes.

Although antigenicity of nucleic acid preparations has been reported,[6-10] none of these preparations could be considered entirely protein-free.

In our preparations the amount of protein, if any, falls beyond the limit of sensitivity of reactions for protein detection (less than 0.1 per cent) and the nitrogen to phosphorus ratios account only for nucleotide nitrogen. However, since the least amount of protein required in a preparation to be antigenic is of the order of 0.1 μg per cent, it is still possible that most or all of the antigenic activity of the RNA preparation might be due to traces of protein.

Because of the potential leukemogenic or oncogenic activity of the RNA fraction and "its" weak antigenicity, it was decided to study the antigenic properties of the protein fraction obtained from fluorocarbon homogenization of tumoral tissue or leukemic cells.

METHODS AND MATERIALS

The preparation of a protein fraction from human leukemic and tumoral cells and control normal tissues derived from the method of homogenization with a fluorocarbon at high speed employed to extract a nucleic acid fraction.[2-4] The latter method was described in full detail before[3, 4] and only the details which pertain to the preparation of the protein fraction will be given here.

Leukemic blood and tumor suspensions were mixed with the fluorocarbon trifluorotrichloroethane* at speeds of 45,000 to 70,000 r.p.m. at 2° C. in air turbine homogenizers.[3, 4] After centrifugation, the homogenate separates in three layers: a supernatant fluid to be re-extracted, a middle gel layer of protein precipitate which is collected, and an undernatant of excess of fluorocarbon which is discarded. The extraction of the supernatant fluid continues until it does not yield middle protein layer any more. Ten to sixteen extractions might be required. The protein layers from the several extractions are mixed and stirred vigorously at 2° C. A result of this stirring is that fluorocarbon is expressed out of its semisolid emulsion with the proteins and can be decanted freely. To the protein is then added chilled 0.15M phosphate buffer, pH 7.2. After resuspending, the mixture is centrifuged in a PR-2 International centrifuge at 1,800 r.p.m. for 10 minutes (2° C.). The supernatant fluid is saved and the precipitate is again vigorously stirred to express more fluorocarbon. The supernatant fluid is added again to the protein gel. This process is continued until no more fluorocarbon can be separated from the protein cake. In the absence of the fluorocarbon about 70 per cent of the protein can be redissolved in the buffered supernatant. The total protein nitrogen is determined and the concentration is adjusted to 0.5 mg. of protein per milliliter.

Protein preparations were obtained by this method from 38 patients with leukemia and 33 individuals with different solid tumors. Of the leukemias 6 were chronic lymphatic in adults and 32 acute, of which 18 were in children (1 of these congenital) and 14 in adults. Of the tumors 29 were carcinomas (26 adenocarcinomas) and 4 sarcomas.

Preparation of Tumoral or Leukemic Antigen.—Tumor tissue obtained at surgical operation is cleaned, finely minced, and suspended in 0.15M phosphate buffer, pH 7.2, in 1:20 weight:volume ratio. To one volume of this suspension or of whole leukemic blood is added one volume of fluorocarbon.

*Genetron No. 113, General Chemical Division, Allied Chemical and Dye Corporation, New York, N. Y.

The protein is separated and the resulting product, labeled "crude tumor extract" (CTE) or "crude leukemic extract" (CLE), is kept at 2° C.

Purification of Crude Extracts.—The crude extracts are absorbed on an antinormal animal serum for partial or total removal of normal antigenic components.

Preparation of Antinormal Animal Serum.—Roller tube primary cultures of human amnion cells are prepared by growing trypsinized amnion cell suspensions (10^6 cells per milliliter) in a medium of NCTC 109 solution and 10 per cent calf serum. Solid sheets of 7- to 10-day-old cultures are scraped off with a Teflon policeman, resuspended in Hanks balanced salt solution, and extracted with fluorocarbon in the manner described for tumoral or leukemic cells. Rabbits (10 pound white New Zealand), goats (110 pound Toggenburg), and donkeys (500 pound Mexican) are injected with this extract of normal amnion cells intravenously on 4 consecutive days and then once a week for a total of 7 injections. Preimmune serum (S_0) and sera of the fifth (S_1), eleventh (S_2), and eighteenth (S_3) day are tested against the antigen by the gel diffusion method indicated below. If antibodies can be demonstrated, the animals are bled one week after the last injection; the serum is quickly separated and frozen.

Absorption of the Crude Tumoral or Leukemic Extract.—Whole serum or the gamma globulin fraction extracted by ethanol have been used for the absorption of normal components from the crude malignant extract. Nine parts of this extract and one part of antinormal rabbit serum, or of the 16 per cent solution of its gamma globulin fraction are mixed, stirred thoroughly, and incubated at 2° C. overnight. The mixture is then centrifuged at 31,000 g for 20 minutes. To the supernatant fluid is then added antinormal rabbit serum (9:1) and the absorption is repeated until, upon centrifugation, the precipitate is reduced to become barely visible. The final supernatant fluid is the purified tumor extract.

Gel Diffusion Method.—The modification of the Ouchterlony technique by Korngold[11] has been adopted. Stainless steel reservoirs replace wells carved in the agar. A distance of 1.8 cm. from the center of the central reservoir to the center of each peripheral reservoir is strictly observed. The plates are prepared and left to "ripen" for about 5 days. It seems that a certain degree of crystallization of the agar promotes quicker and more uniform diffusion. The reservoirs are refilled after 2 or 3 days. The plates are incubated at 25° C. and read daily from the fifth to the tenth day.

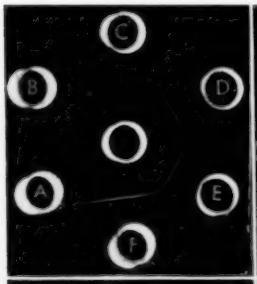
The immunization of animals with purified tumoral or leukemic extract followed the same scheme presented for the production of antinormal serum. The dose is 0.1 mg. of protein per pound of body weight.

RESULTS

The production of antibodies in the animals against normal or malignant extracts is indicated by the appearance of lines in the agar plates. Since visible precipitation in the agar depends on a minimal fixed amount of antibody, the lines are not essentially different in the different sera (S_1, S_2, S_3, S_4) collected during an immunization period (Fig. 1). That minimal amount may be determined by serial dilution of the sera.

Normal amnion culture extract produces several lines against its homologous antisera (Fig. 2). Several lines are also shown when crude tumoral or leukemic extract is matched against antinormal serum (Fig. 3). When antitumoral or leukemic sera from different rabbits were used against crude tumoral or leukemic extracts a reaction of identity revealed that several antibodies are present but that different animals produced the same antibodies (Fig. 4).

Fig. 1.



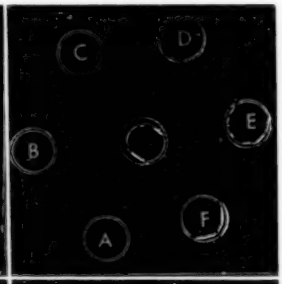
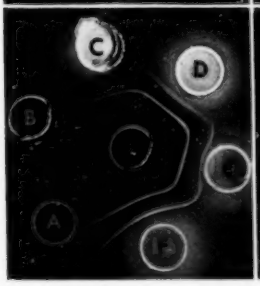


Fig. 2.

Fig. 3.



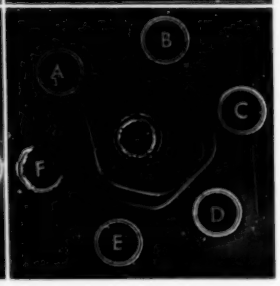


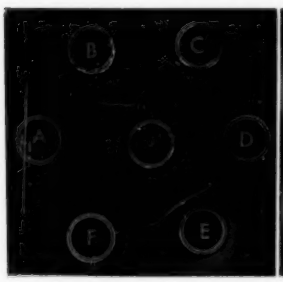
Fig. 4.

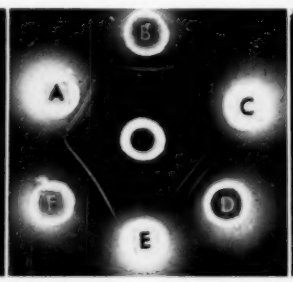
Fig. 1.—In the center (unmarked) is the antigen, a crude leukemic extract (acute leukemia). In the peripheral reservoirs samples of sera from the same rabbit, injected with this antigen, taken at the 0 *(B)*, fifth *(C)*, eleventh *(D)*, eighteenth *(E)*, and twenty-fifth *(F)* days. *A*, Saline. A reaction of identity indicates that the antibodies are the same in the different samples.

Fig. 2.—In the center normal human amnion extract. Periphery: *D*, *E*, and *F*. Antisera of three different rabbits prepared with the antigen in the center. *A*, *B*, *C* reservoirs are empty. Rabbits *D* and *E* produced 2 antinormal antibodies (identical). Rabbit *F* produced a third one (3 lines).

Fig. 3.—In the center crude leukemic extract (acute leukemia). In periphery: *A*, saline; *B*, *C*, *D*, *E*, *and F*, antinormal serum samples from different rabbits. Reaction of identity for all antibodies (antinormal).

Fig. 4.—In the center: Crude leukemic extract (acute leukemia). *A*, Saline. *B*, *C*, *D*, *E*, *F*, Antisera from five different rabbits prepared with the center antigen.





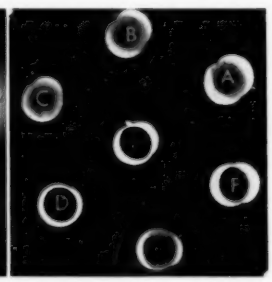


Fig. 5. Fig. 6. Fig. 7.

Fig. 5.—In the center purified leukemic extract (the same as Fig. 4 after complete absorption on antinormal rabbit serum). At the periphery antisera from five different rabbits prepared with the purified leukemic extract. Only one line of isoprecipitation is left indicating the presence of a specific leukemic antigen independent from group, type, or cell-specific normal antigens.

Fig. 6.—In the center an antiserum prepared in a rabbit with a crude tumoral extract (carcinoma of the breast). *A*, The crude tumoral extract showing 4 lines. *B*, The same extract after the first step of absorption on antinormal rabbit serum shows 3 lines only. *C* and *D*, After another step only 2 lines (the lines in *C*, though blurred in the shadowgraph, were clearly visible in the plate). *E*, Saline. *F*, After complete absorption by an excess of antinormal saline there is a persistent line.

Fig. 7.—Center, purified tumoral extract. *A*, *F*, *E*, Rabbit antisera prepared with the antigen in the center. *D*, *C*, *B*, Goat antisera prepared with the same antigen. Although the goat did not produce antibodies against this antigen, there is a line of heterophil antibody between the rabbit *(E)* and the goat *(D)* sera.

After complete absorption by antinormal rabbit serum or its gamma globulin, the purified tumoral or leukemic extract gives only one line of isoprecipitation with antisera from different rabbits prepared with the same purified malignant extract (Fig. 5), which indicates a single antibody that is the same in different rabbits. During fractionated absorption the different lines belonging to normal components can be eliminated one by one until one persists that is not absorbed by an excess of antinormal serum (Fig. 6). These

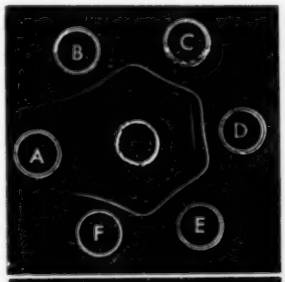


Fig. 8.

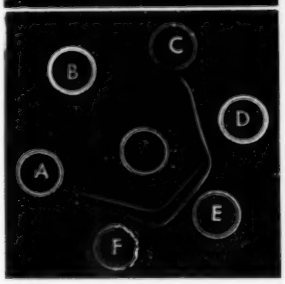


Fig. 9.

Fig. 8.—Center, Normal amnion extract. Periphery sera from different rabbits (*B, C, D, E, F*) injected with the same extract. *A*, Saline. Some rabbits show more antibodies than did others.

Fig. 9.—Center, Normal amnion extract. *A*, Preimmune serum in rabbit (S_0). *B*, Serum of fifth day (S_1). *C*, Serum of eighth day (S_2). *D*, Serum of eleventh day (S_3). *E*, Serum of eighteenth day (S_4). *F*, Serum of twenty-fifth day (S_5).

results also indicate that such lines are not due to isoantigens from traces of rabbit serum left after absorption of the crude tumoral extract. Occasionally lines of isoantigens were found between two contiguous reservoirs which contained sera from different animals even when there was a reaction of identity relative to the center well (Fig. 7). Less well explained is an occasional reaction of unidentity between antisera from different rabbits and normal extract (Fig. 8). Multiple antibodies develop progressively during the course of immunization (Fig. 9).

When the crude tumoral extract is matched against antisera prepared with the purified tumoral extract again one line is obtained. When purified tumoral extract is mixed and incubated with antinormal rabbit serum it still gives one line of reaction. However, if the same purified tumoral extract is mixed previously with a rabbit antiserum prepared with purified tumoral extract and then checked against the same serum no lines of reaction occur (Table I).

TABLE I. PRECIPITATION IN GEL DIFFUSION

ANTIGENS	ANTISERA IN RABBITS			
	ANTI-CTE (LINES)	ANTI-CLE (LINES)	ANTI-PTE (LINES)	ANTI-PLE (LINES)
Crude tumoral extract (CTE)	Several	1	1	None
Crude leukemic extract (CLE)	1	Several	None	1
Purified tumoral extract (PTE)	1	1	1	None
Purified leukemic extract (PLE)	1	1	None	1

The same phenomena are observed whether the extracts are from carcinomas or sarcomas. When leukemic extracts are used instead of tumoral extracts the same general phenomena regarding normal antigens in crude extracts are observed. However, in purified extracts reactions of unidentity revealed differences between tumors and leukemias and, within the leukemias, between acute stem cell and chronic lymphatic leukemias (Table II).

TABLE II. PRECIPITATION IN GEL DIFFUSION

ANTIGENS, PURIFIED EXTRACTS	ANTISERA IN RABBITS			
	ANTI-CA (LINES)	ANTI-SA (LINES)	ANTI-AL (LINES)	ANTI-CL (LINES)
Carcinomas (CA)	1*	1*	None	None
Sarcomas (SA)	1*	1*	None	None
Acute stem cell leukemia (AL)	None	None	1†	None
Chronic lymphatic leukemia (CL)	None	None	None	1†

*This line can be eliminated after absorption with either anti-CA or anti-SA sera.
†This line can be eliminated only after absorption with the specific serum.

The results of these tests tend to indicate that:

1. In a crude leukemic or tumoral extract there are, as expected, several antigens. Although usually more than one antigen is associated with normal components, only one antigen could be demonstrated in tumoral or leukemic extracts purified by extensive and fractionated absorption with antinormal animal serum. This is in agreement with the generally accepted notion of the antigenic simplification that accompanies in vivo and in vitro development of a malignant condition of a cell line.

2. The antigens which remain after absorption in a tumoral extract are not the same as the antigens which remain after absorption in a leukemic extract.

3. While no differences were uncovered between antigens from the carcinomas and sarcomas studied, there were differences between acute stem cell and chronic lymphatic leukemias.

DISCUSSION

After many unsuccessful attempts to separate group specific from tumor specific antigens[8, 12-16] and the diametrically opposite findings of Seligman and Weiler and of Bjørklund[17-19] reviewed by Lennette[20] and Zilber,[21] workers started to accumulate positive data on the problem when carefully controlled experiments with the method of anaphylactic desensitization reaction in the guinea pig led Zilber,[21] Sherschulskaya,[22] and Parnes[23] to conclude that in

different forms of carcinomas, sarcomas, and leukemias antigens can be demonstrated which do not occur in the normal tissue counterparts. Differences were found between lymphatic and stem cell leukemia[23] but, contrary to our findings, differences could also be found between different kinds of solid tumors and even between carcinomas of different localizations.[21]

Attempts have also been made to separate both in antigenic mixtures and in antisera the antigens or antibodies of, or to, normal components from the cancer specific ones. Mann and Welker[24] produced rabbit antisera with extracts of human tumors previously absorbed on alumina. Advantage was taken of the apparent fact that precipitins to normal components disappeared from the animal blood 2 months before the titer of anticancer precipitins started to decline. Kosyakov and co-workers[25] eliminated from tumor antisera antibodies against normal antigens by absorbing the antisera with formalin-fixed normal splenic tissue. Korosteleva and associates[26] used 80 per cent glycerol to fix the normal tissues for absorption of antitumor sera. Parnes,[23] using antitumor sera prepared in goats and donkeys, showed, by means of a gel diffusion method, that human leukemic cells contain antigens different from those of normal leukocytes and concluded that there is a simplification of the normal antigenic pattern (2 lines versus 3 lines) and acquisition of new antigenic properties (nonidentity reaction with lines from normal antigens). Calaresu and coauthors[27] had indicated that in chronic lymphatic leukemia there is a specific antigen adsorbed on the erythrocytes, a finding confirmed and extended to other forms of leukemias and reticuloses by Parnes and Lacour.[28] Taylor and collaborators[29] have used fluorocarbon to extract complement fixing antigens from human neoplastic tissues and report that "host tissue complement-fixing antigens could be removed from crude tumor suspension, leaving behind apparently specific antigens." Although we could still demonstrate host tissue antigens in our tumor-fluorocarbon extracts, it is conceivable that the success in complete removal of normal antigens by antinormal serum was possible because of the previous partial removal by the fluorocarbon treatment.

A comparative study of antigenic properties of cancer tissue and of normal organs was undertaken by Kosyakov and Kuznetsova.[30] The antigens were prepared from cancerous and normal tissues of 18 cadavers of cancer victims to ensure isogenetic homogeneity. Antitumor antisera was prepared in rabbits, goats, and horses. Complement fixation tests showed that cancerous tissues contain "specific antigens" which they alone possess and which are not found in noncancerous tissue of the same individual. These "cancer antigens" differ immunologically from normal (group, type, organo specific) antigens and appear in the organism during the development of the cancer process. The characteristic properties of the cancer antigens do not depend upon the location of the cancer or upon the specificity of the normal antigens that the tumor contains (fibrin, collagen, endothelium of vessels, connective tissue elements or others). Using similar methods Korosteleva and Kosyakov[31] were able to distinguish between cancer antigens of different human tumors and conclude that there is no single antigen common to all tumors but instead there is an antigenic tumor individuality, a conclusion we could not confirm.

The method of absorbing an antigenic mixture of normal and tumoral antigens, as it exists in a tumor extract, with an antinormal serum, has the advantage over the method of absorbing antinormal antibodies in an antiserum with normal tissue extracts that the antitumor potency of the antiserum is not reduced by absorption and that the absorption of the antigenic mixture does not reduce appreciably the antigenicity of the remaining components.

The possibility of preparing a human antileukemic or antitumoral antigen by fluorocarbon extraction followed by differential absorption on antinormal animal serum, and thus of preparing pure antitumor sera, enables us to study the chemical and immunologic characteristics of these antigens and the protective and therapeutic capacities of the respective antibodies as well as the opportunity to stimulate, through active immunization, the natural resistances of the host organism to the malignant disease.

SUMMARY

Absorption of fluorocarbon extracts from human tumoral and leukemic cells with antinormal animal serum prepared with fluorocarbon extracts of human amnion resulted in a purified tumoral and leukemic antigen preparation. Antisera in animals against this purified antigen contained no demonstrable antibodies against normal tissue antigens. Antigenic differences were found between solid tumors and leukemias and between chronic lymphatic and acute stem cell leukemias.

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EFFECTS OF GLUCAGON ON PLASMA UNESTERIFIED FATTY ACIDS AND IN NITROGEN METABOLISM

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The injection of glucagon into subjects in a fasting state resulted in a rise of plasma unesterified fatty acids 4 to 6 hours after injection. This increase was significantly greater than that produced by fasting alone. Glucagon administration resulted in a negative nitrogen balance in 5 subjects. This was shown to be independent of the adrenal-pituitary axis. When glucagon was administered to 2 obese women who were receiving an inadequate caloric intake, a negative nitrogen balance ensued. This suggested that the catabolic effect of glucagon was not mediated via glycogenolysis and consequent gluconeogenesis. From these and other data, it was proposed that glucagon increased the peripheral caloric demand, thereby leading to increased levels of unesterified fatty acids and the negative nitrogen balance.

THE only clearly documented action of glucagon is the acceleration of hepatic glycogenolysis. It has been asserted by some[1, 2] that glucagon promotes the peripheral utilization of glucose. However, others[3, 4] have concluded that it has no effect on this process. Relatively few studies have been designed to yield information regarding the effects of glucagon on fat and protein metabolism in man.

Certain aspects of these phases of the metabolic response to glucagon have been examined in this study. The effect of glucagon on plasma unesterified fatty acid (UFA) levels was studied as one parameter of fat metabolism. Protein metabolism was examined by balance techniques during chronic administration of glucagon.

METHODS

The acute effects of glucagon on plasma UFA levels were analyzed in 9 subjects. One milligram of crystalline glucagon‡ was injected intravenously and the blood glucose and plasma UFA concentrations were determined at 0, 1, 2, 4, and 6 hours after injection. A similar test was conducted in each subject using a saline injection either 1 week before or 1 week after the injection of glucagon. The injections were made between 8:00 A.M. and 9:00 A.M., after a 16 hour fast. Food was not eaten during the tests. The blood glucose and plasma UFA levels were analyzed by the methods of Folin and Wu[5] and Gordon,[6] respectively.

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The balance studies were conducted using standard metabolic balance techniques. The subjects were ambulatory. Each experimental period was interposed between appropriate control periods. Sample diets were analyzed at least twice during each study. Glucagon was given in varying doses at 3 to 6 hour intervals either subcutaneously or intramuscularly. Urines were analyzed daily for nitrogen, phosphorus, calcium, sodium, potassium, chloride, creatinine, Porter-Silber chromogens, and alpha amino acid nitrogen by methods previously reported.[7] Fecal electrolytes were analyzed in 6 day pools. Serum concentration of sodium, chloride, potassium, urea nitrogen, calcium, phosphorus, and CO_2 content were determined by the Clinical Pathology Department of the Clinical Center. The theoretic phosphorus balances have been calculated using a calcium to phosphorus ratio of 2:1 and a nitrogen to phosphorus ratio of 15:1.[8]

SUBJECTS

The group given the intravenous injections of glucagon comprised 7 normal adults and 2 patients who had undergone hypophysectomy for control of metastatic cancer. These 2 patients were in relatively good health and were receiving 37.5 mg. of cortisone acetate and 50 mcg. of triiodothyronine daily during the tests. All subjects were receiving an adequate caloric intake.

Five patients were studied on a metabolic balance regimen.

A.M. was a 48-year-old white woman with metastatic breast cancer. The patient was clinically well and the only evidence of disease was two osteoblastic lesions of the hip. Liver function tests were normal. She received 2,200 calories daily and was given 0.25 mg. glucagon subcutaneously every 4 hours.

M.H. was a 52-year-old white man referred because of hypoglycemia and recurrent intra-abdominal sarcoma. The only clinical evidence of cancer was a mass in the left upper quadrant. To prevent hypoglycemic attacks, food was needed at 3 hour intervals. He received 3,700 calories daily and was given 1 mg. glucagon intramuscularly every 3 hours for 3 days, followed by 2 mg. glucagon intramuscularly every 3 hours for 3 days.

M.S. was a 22-year-old white woman who had been subjected to pituitary stalk section at another hospital because of metastatic choriocarcinoma. At the time of the study and after amethopterin therapy she was apparently free of disease. The serum protein-bound iodine level was 2.4 μg per 100 ml. and urinary Porter-Silber chromogens were 0.5 to 1.0 mg. per 24 hours. Throughout the study, she received 10 mg. of cortisone acetate and 50 mcg. of triiodothyronine daily. Glucagon was given initially, 1 mg. every 4 hours intramuscularly, but anorexia and nausea necessitated a reduction of the dose to 0.25 mg. every 4 hours on the second day and 0.1 mg. every 4 hours on the sixth day.

E.R. was a 40-year-old obese white woman with diabetes of 1 year's duration who did not require insulin. She was referred for evaluation of possible Cushing's syndrome. Tests of adrenal function were normal. The patient was given a 1,030 calorie diet and received 1.0 mg. glucagon subcutaneously every 4 hours for the first 2 days and 0.5 mg. every 4 hours for 4 days.

A.L. was a 46-year-old obese white woman with diabetes of 8 years' duration referred for evaluation of possible Cushing's syndrome. Tests of adrenal function were normal. Home management of diabetes consisted of 15 to 20 units of protamine zinc insulin daily and an attempt at dietary control. During the first study, she received 800 calories daily and no insulin. Glucagon was given at a dose of 0.25 mg. every 6 hours subcutaneously for 5 days and 1.0 mg. every 6 hours on the sixth day. During the second study 1 month later, the patient received 2,500 calories and 15 units of protamine zinc insulin daily. Glucagon was injected in a dose of 0.25 mg. every 6 hours subcutaneously.

RESULTS

After the intravenous injection of glucagon, the average blood glucose concentrations were significantly increased at 1, 2, and 4 hours when compared

TABLE I. BLOOD GLUCOSE (MG. PER CENT)

PATIENT	0 HR.		1 HR.		2 HR.		4 HR.		6 HR.	
	C	G	C	G	C	G	C	G	C	G
1	81	83	80	163	81	165	86	175	92	115
2	86	87	87	102	84	87	84	92	93	90
3	93	88	87	108	88	85	87	88	85	90
4	103	96	100	138	104	150	108	190	120	165
5	123	144	122	270	120	143	100	135	127	140
6	100	119	92	124	112	115	112	122	114	123
7	100	100	95	145	88	95	116	121	107	130
8	80	74	73	72	75	95	100	80	94	90
9	87	103	86	100	92	114	87	128	89	130
Average	95	89	91	136	95	117	100	126	105	119
	$p = 0.5^*$		$p < 0.02$		$p < 0.05$		$p < 0.05$		$p > 0.2$	

C = control; G = glucagon.

*Because the variances of the control and experimental groups differed, statistical significance was tested by the Welch-Aspin t test.[21]

TABLE II. PLASMA UFA (μEq/L.)

PATIENT	0 HR.		1 HR.		2 HR.		4 HR.		6 HR.	
	C	G	C	G	C	G	C	G	C	G
1	860	800	610	290	760	820	1,000	1,400	1,100	1,750
2	580	590	430	280	700	370	1,350	1,610	1,150	1,300
3	410	390	330	140	390	220	430	1,100	480	800
4	250	320	300	160	320	350	410	720	560	880
5	620	580	1,020	460	910	350	1,020	840	840	1,060
6	400	320	360	220	470	620	810	1,280	460	1,600
7	250	250	320	80	330	500	400	810	740	890
8	630	520	570	250	930	480	860	610	710	790
9	420	410	510	220	690	140	640	1,140	770	600
Average	490	450	490	240	610	430	770	1,060	760	1,100
	$p > 0.5^*$		$0.02 > p > 0.01$		$p > 0.3$		$0.1 > p > 0.05$		$0.1 > p > 0.05$	

C = control; G = glucagon.

*Because the variances of the control and experimental groups differed, statistical significance was tested by the Welch-Aspin t test.[21]

with the averages of the control values (Table I). The blood sugar level did not change after the saline injection.

The effect of glucagon on UFA levels is recorded in Table II. One hour after injection of glucagon, the average plasma UFA level was 240 μEq per liter compared with the initial value of 400 μEq per liter. This decrease was significant ($p < 0.02$) when compared to the 1 hour value of the groups with injected saline. By the second hour, the average UFA level in the group receiving glucagon had returned to the base-line level. However, at the fourth and sixth hours, the average plasma UFA levels were 950 and 1,100 μEq per liter, respectively, compared with the control levels of 770 and 760 μEq per liter. Because of the wide variations in the control group, the "p" values lay between 0.05 and 0.1.

In Fig. 1, the increments in plasma UFA concentrations which resulted from the glucagon injection have been plotted. The shaded area is the range of the increments of UFA concentration after saline injection and the heavy line connects the averages of these values. When the individual tests are considered, the UFA levels 4 and 6 hours after injection of glucagon exceeded those of the saline control in 15 of 18 instances (Table III). The average increase

of UFA concentration from the initial values at both the 4 and 6 hour periods was more than 10 times the standard error of the analysis. In spite of the large variability of response, statistical tests of significance revealed differences between the 2 groups at 4 and 6 hours ($p < 0.05$). There was no significant correlation between the increments in glucose levels and UFA levels although the higher glucose levels tended to be associated with the higher UFA levels at the 4 and 6 hour periods.

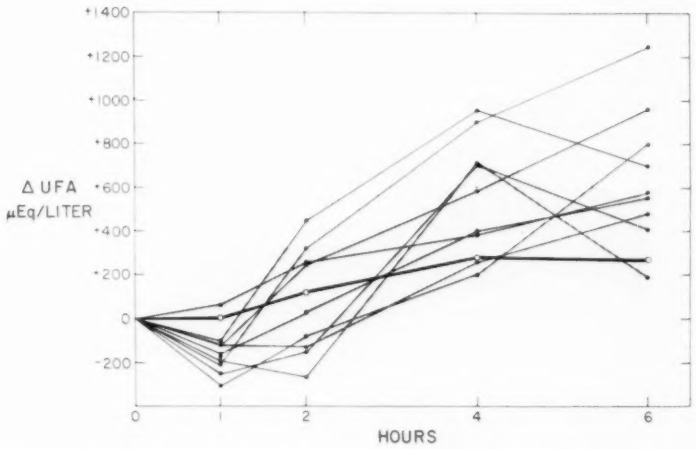

Fig. 1.—Increments in plasma UFA after injection of glucagon. The shaded area encompasses the range of the responses of the control group. The heavy black line connects the average values in this group.

BALANCE STUDIES

The data pertaining to nitrogen and phosphorus excretion are presented in Table IV. The balance data relating to calcium, sodium, potassium, and chloride have been condensed in Table V since there were no consistent changes in the balances of these electrolytes. Serum levels of sodium, potassium, chloride,

TABLE III

	Δ UFA (4 HR.) (μEq/L.)			Δ UFA (6 HR.) (μEq/L.)		
	C	G	G–C	C	G	G–C
	140	600	+460	240	950	+710
	770	1,020	+350	570	710	+140
	20	710	+690	70	310	+240
	160	400	+240	310	560	+250
	400	260	−140	220	480	+260
	410	960	+550	60	1,280	+1,220
	150	560	+410	490	640	+150
	230	190	− 40	80	270	+190
	220	730	+510	350	190	−160
Average	280	600	+320	260	600	+340
		$0.05 > p > 0.02$			$0.02 > p > 0.01$	

Because the variances of the control and experimental groups differed, statistical significance was tested by the Welch-Aspin t test.[23]

TABLE IV

PATIENT	DAY	NITROGEN*			PHOSPHORUS*		
		URINE	STOOL	BALANCE	URINE	STOOL	BALANCE
A. L.	1	8.3	0.6	+1.5	0.48	0.20	+0.23
H	2	9.7	0.6	+0.1	0.73	0.20	−0.02
	3	7.8	0.6	+2.0	0.56	0.20	+0.15
	4	8.0	0.6	+1.8	0.52	0.20	+0.19
	5	8.3	0.6	+1.5	0.53	0.20	+0.18
	6	8.8	0.6	+1.2	0.55	0.20	+0.16
	1	9.2	0.8	+0.4	0.68	0.37	−0.07
	2	8.8	0.8	+0.8	0.62	0.37	−0.01
	3	9.7	0.8	−0.1	0.64	0.37	−0.03
	4	9.8	0.8	−0.2	0.64	0.37	−0.03
	5	9.8	0.8	−0.2	0.56	0.37	−0.05
	6	9.7	0.8	−0.1	0.48	0.37	+0.13
	1	7.5	0.6	+2.1	0.35	0.20	+0.09
	2	8.3	0.6	+1.5	0.40	0.20	+0.04
	3	8.3	0.6	+1.5	0.54	0.20	−0.10
	4	7.9	0.6	+1.9	0.56	0.20	−0.12
	5	8.7	0.6	+1.1	0.66	0.20	−0.22
	6	9.3	0.6	+0.5	0.68	0.20	−0.24
M. H.	1	17.4	1.6	−1.4	0.82	1.16	+0.15
	2	14.6	1.6	+1.4	0.83	1.16	+0.14
	3	14.2	1.6	+1.8	0.79	1.16	+0.18
	4	13.9	1.6	+2.1	0.73	1.16	+0.24
	5	13.9	1.6	+2.1	0.90	1.16	+0.07
	6	14.0	1.6	+2.0	0.97	1.16	0
	1	17.5	2.1	−2.0	1.19	1.31	−0.37
	2	20.3	2.1	−4.8	1.19	1.31	−0.37
	3	21.2	2.1	−5.7	1.09	1.31	−0.27
	4	20.6	2.1	−5.1	1.17	1.31	−0.35
	5	19.2	2.1	−3.7	1.07	1.31	−0.25
	6	19.3	2.1	−3.8	0.87	1.31	−0.05
	1	15.4	0.8†	+0.2	0.80	1.24‡	+0.09
	2	17.1	0.8	−1.3	0.71	1.24	+0.18
	3	12.3	0.8	+3.5	0.75	1.24	+0.14
	4	13.5	0.8	+2.3	0.85	1.24	+0.04
	5	12.7	0.8	+3.1	0.92	1.24	−0.03
	6	14.0	0.8	+1.8	0.93	1.24	−0.04
E. R.	1	8.9	2.7	−0.3	0.68	0.19	+0.06
	2	9.0	2.7	−0.4	0.68	0.19	+0.06
	3	8.6	2.7	0	0.80	0.19	−0.06
	4	9.2	2.7	−0.6	0.67	0.19	+0.07
	5	9.4	2.7	−0.8	1.17	0.19	−0.43
	6	8.8	2.7	−0.2	0.67	0.19	+0.07
	1	11.5	2.9	−3.1	0.79	0.18	−0.04
	2	11.8	2.9	−3.4	0.75	0.18	0
	3	10.3	2.9	−1.9	0.62	0.18	+0.13
	4	10.2	2.9	−1.8	0.49	0.18	+0.26
	5	10.0	2.9	−1.6	0.76	0.18	−0.01
	6	8.2	2.9	+0.2	0.56	0.18	+0.19
	1	7.4	2.8	+1.1	0.48	0.30	+0.15
	2	7.7	2.8	+0.8	0.70	0.30	−0.07
	3	6.6	2.8	+1.9	0.82	0.30	−0.19
	4	7.4	2.8	+1.1	0.51	0.30	+0.12
	5	7.9	2.8	+0.6	0.64	0.30	−0.10
	6	6.8	2.8	+1.7	0.62	0.30	+0.10

*All values are in grams per 24 hours.

†Stool lost; values are averages of previous two collections.

‡Intake 8.2 Gm. nitrogen; 1.02 Gm. phosphorus.

§Intake 7.8 Gm. nitrogen; 0.94 Gm. phosphorus. Vomitus 2.1 Gm. nitrogen; 0.14 Gm. phosphorus.

TABLE IV—CONT'D

PATIENT	DAY	NITROGEN			PHOSPHORUS		
		URINE	STOOL	BALANCE	URINE	STOOL	BALANCE
M. S.	1	11.2	1.1	−1.1	0.71	0.96	−0.09
	2	11.2	1.1	−1.1	0.74	0.96	−0.12
	3	11.3	1.1	−1.2	0.86	0.96	−0.24
	4	10.7	1.1	−0.6	0.95	0.96	−0.33
	5	11.2	1.1	−1.2	0.88	0.96	−0.26
	6	11.5	1.1	−1.3	0.79	0.96	−0.17
	1	13.0	1.2	−3.0	1.15	0.96	−0.53
	2†	11.9	1.2	−4.8	0.92	0.96	−0.66
	3	12.1	1.2	−2.1	0.74	0.96	−0.12
	4	12.4	1.2	−2.4	0.78	0.96	−0.16
	5§	9.9	1.2	−5.4	0.85	0.96	−0.81
	6	8.8	1.2	+1.2	0.57	0.96	−0.05
	7	12.2	1.2	−2.2	0.68	0.96	−0.16
	8	13.9	1.2	−3.9	0.83	0.96	−0.23
	9	14.1	1.2	−4.1	0.84	0.96	−0.24
	1	11.6	1.6	−2.0	0.79	0.76	+0.07
	2	9.5	1.6	+0.1	0.59	0.76	+0.23
	3	10.2	1.6	−0.6	0.60	0.76	+0.22
A. M.	1	9.4	1.1	−0.8	0.69	0.28	+0.13
	2	9.7	1.1	−1.1	0.67	0.28	+0.15
	3	8.8	1.1	−0.2	0.76	0.28	+0.06
	4	9.2	1.1	−0.6	0.74	0.28	+0.08
	5	9.5	1.1	−0.9	0.70	0.28	+0.12
	6	8.6	1.1	0	0.63	0.28	+0.19
	1	11.6	1.2	−3.1	0.84	0.27	−0.01
	2	9.6	1.2	−1.1	0.75	0.27	+0.08
	3	11.2	1.2	−3.1	0.86	0.27	−0.03
	4	9.8	1.2	−1.3	0.80	0.27	+0.03
	5	10.5	1.2	−2.0	0.80	0.27	+0.03
	6	12.5	1.2	−4.0	0.90	0.27	−0.07
	1	6.7	1.1	+1.9	0.49	0.26	+0.35
	2	7.0	1.1	+1.6	0.54	0.26	+0.30
	3	7.6	1.1	+1.0	0.59	0.26	+0.25
	4	8.0	1.1	+0.6	0.68	0.26	+0.16
	5	8.5	1.1	+0.1	0.72	0.26	+0.12
	6	7.8	1.1	+0.8	0.59	0.26	+0.09
A. L. I	1	7.7	0.7	+0.6	0.59	0.18	+0.04
	2	9.9	0.7	−1.6	0.68	0.18	−0.05
	3	9.3	0.7	−1.0	0.69	0.18	−0.06
	4	10.2	0.7	−1.9	0.75	0.18	−0.11
	5	9.7	0.7	−1.4	0.65	0.18	−0.02
	6	9.4	0.7	−1.1	0.69	0.18	−0.06
	1	9.4	1.0	−1.4	0.66	0.22	−0.16
	2	9.0	1.0	−1.0	0.69	0.22	−0.19
	3	10.4	1.0	−2.4	0.59	0.22	−0.09
	4	9.2	1.0	−1.2	0.41	0.22	+0.06
	5	9.6	1.0	−1.6	0.62	0.22	−0.12
	6	10.2	1.0	−2.2	0.58	0.22	−0.08
	1	7.3	0.6	+0.7	0.42	0.09	+0.05
	2	8.3	0.6	+0.1	0.52	0.09	+0.10
	3	9.2	0.6	−0.8	0.57	0.09	+0.05
	4	7.7	0.6	+0.6	0.53	0.09	+0.09
	5	7.8	0.6	+0.6	0.44	0.09	+0.18
	6	5.9	0.6	+2.5	0.64	0.09	−0.02

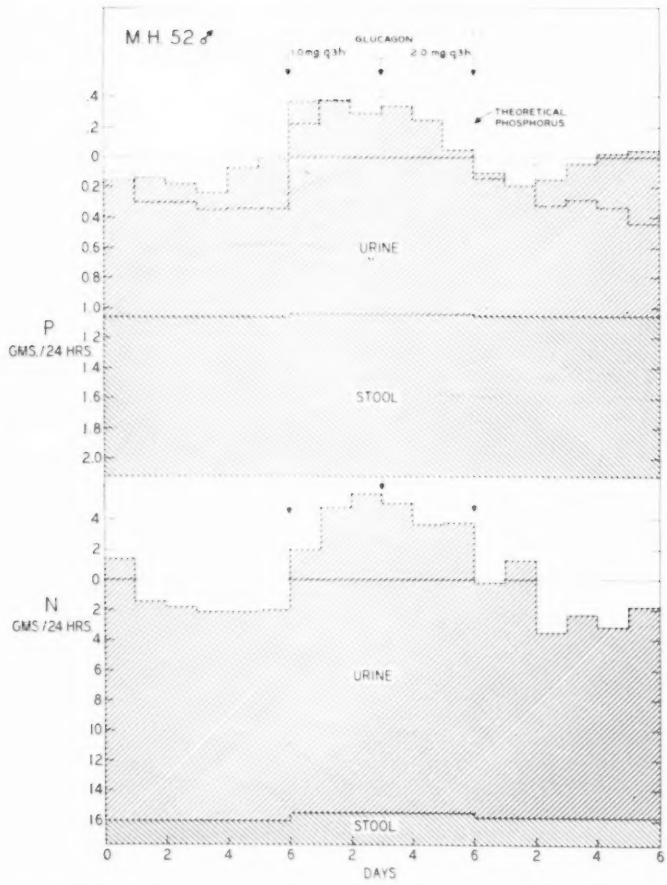

Fig. 2.—Effect of glucagon on nitrogen and phosphorus balance. (All balance data have been plotted according to the method of Reifenstein and associates.[8] Intakes are plotted from the zero line down and fecal and urinary values are superimposed on this. A shaded area above the zero line indicates a negative balance.)

calcium, phosphorus, and urea nitrogen have not been presented since all were within the normal range initially and did not change with the administration of glucagon. There were no changes in the excretion of Porter-Silber chromogens, amino acid nitrogen, or creatinine.

The administration of glucagon to A.M. increased the urinary nitrogen excretion by an average of 1.7 Gm. daily. Phosphorus excretion likewise increased. The blood glucose levels were elevated to 130 mg. per 100 ml. 3 hours after the injections.

In M.H. injection of glucagon resulted in a negative nitrogen balance of 25.1 Gm. in 6 days. Phosphorus excretion was proportionately increased (Fig. 2) and the urinary potassium was likewise elevated (Table IV). Doubling the dose of glucagon during the latter 3 days did not lead to a further increase in nitrogen excretion, which suggested that a maximally effective dose had been attained. Because of the feeding at 3 hour intervals, it was difficult to assess

the effect of glucagon on the blood sugar levels. These, however, averaged 81 and 67 mg. per 100 ml. at 1 and 3 hours after a feeding during the control periods. With the chronic administration of glucagon, the blood glucose levels averaged 146 and 88 mg. per 100 ml. at comparable times.

The balance study of M.S. was less satisfactory because anorexia and nausea necessitated decreasing the glucagon dose on 2 occasions and resulted in poor food intake on 2 days. However, on the first day of the period of glucagon administration and again during the last 3 days, the increase in urinary nitrogen is evident (Table IV). Blood sugar levels 1 and 3 hours postprandially averaged 201 and 115 mg. per 100 ml. compared to control levels of 130 and 100 mg. per 100 ml., respectively.

In A.L. we attempted to analyze the effects of glucagon when liver glycogen had presumably been reduced by an inadequate caloric intake and subsequently when it could be expected to be increased. During the first study period, she received no insulin and an 800 calorie diet. This resulted in a weight loss of 3.0 kilograms during the 18 days. There were only traces of sugar in the urine. During the initial control period, the nitrogen balance averaged −1.1 Gm. daily (Fig. 3). Six days of glucagon administration resulted in an average nitrogen balance of −1.6 Gm. daily. This change is within the cumulative error of the balance technique. However, during the subsequent control period, the patient retained nitrogen, which suggested a compensatory anabolic phase after a catabolic episode. It is also possible that the base line of nitrogen excretion shifted as continuous readjustments to the low caloric intake became operative. The phosphorus balance (Table IV) became negative with glucagon

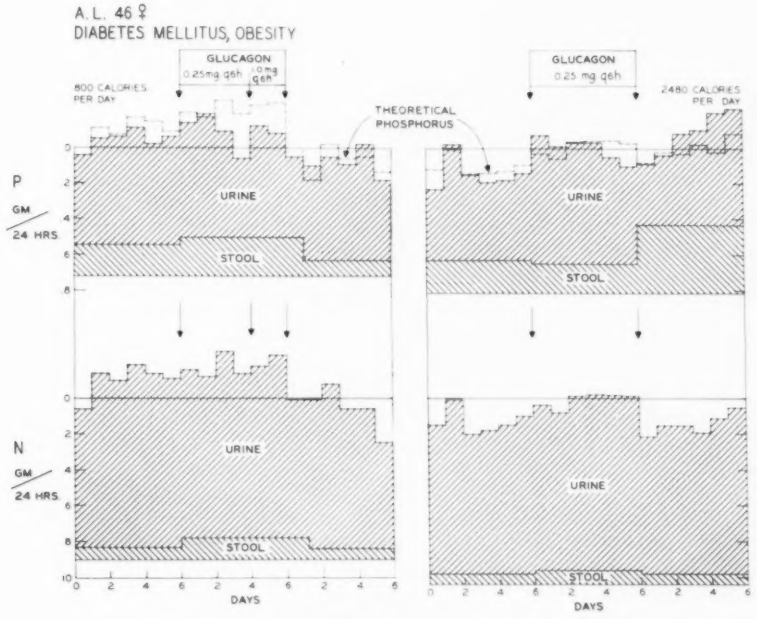

Fig. 3.—Effect of glucagon on nitrogen and phosphorus balance.

TABLE V

PATIENT	DAYS IN PERIOD	WEIGHT AT START OF PERIOD (KG.)	CALCIUM (GM./PERIOD)			SODIUM (MEQ./PERIOD)			CHLORIDE (MEQ./PERIOD)			POTASSIUM (MEQ./PERIOD)		
			URINE	STOOL	BALANCE	URINE	STOOL	BALANCE	URINE	STOOL	BALANCE	URINE	STOOL	BALANCE
M. H.	6	68.0	0.52	9.4	0	558	7	+27	596	15	−17	396	84	+66
	6	68.0	0.39	9.6	−0.1	544	7	+31	545	13	+26	423	120	+ 3
	6	67.8	0.57	—	—	502	—	—	675	—	—	372	—	—
		68.2												
M. S.	6	47.1	1.3	6.9	−1.4	718	14	−42	622	4	−26	388	66	+14
	9	47.3	2.2	9.6	−2.5	974	22	−51	937	6	−43	594	99	+ 9
	3	47.6	0.5	4.2	−1.5	274	13	+28	215	6	+79	163	45	+16
		47.7												
A. L. I	6	96.1	0.35	0.66	−0.1	122	12	+18	165	7	0	251	33	−26
	6	95.2	0.48	1.26	−0.8	161	16	−24	178	9	+30	273	46	−18
	6	94.4	0.45	0.60	−0.3	131	10	+11	132	6	+24	195	37	+26
		93.1												
A. L. II	6	90.4	0.84	0.44	+0.2	89	18	+61	120	14	+12	288	—	—
	6	90.6	1.10	0.52	−0.1	128	18	+22	109	6	+31	274	—	—
	6	90.5	1.07	1.2	−0.8	44	108	+16	106	12	+28	242	—	—
		90.3												
E. R.	6	108.6	0.84	0.60	−0.2	140	18	+28	—	—	—	206	54	+40
	6	107.5	0.75	0.66	−0.2	120	12	+54	—	—	—	240	72	− 6
	6	106.0	0.89	0.90	−0.6	—	18	—	—	—	—	175	102	+29
		104.8												
A. M.	6	64.3	0.64	1.5	+0.22	162	6	+20	157	9	+12	270	42	+22
	6	64.1	0.60	1.4	+0.36	149	22	+17	149	9	+20	314	48	−28
	6	63.0	0.72	1.4	+0.24	184	8	− 4	168	4	+ 6	266	44	+24
		63.2												

administration, which again suggested a catabolic phase. Blood sugar levels before glucagon administration and 1 and 2 hours after the injections did not appear to differ from comparable values in the control periods.

In the second study, the patient received 2,380 calories daily and 15 units protamine zinc insulin daily. This led to a positive nitrogen balance during the control periods (Fig. 3), but there was no net weight change over the duration of the study. Glucagon administration resulted in a slight negative nitrogen balance, but the algebraic difference between the initial control period and the glucagon period averaged −1.2 Gm. daily. An anabolic phase again followed glucagon administration. The blood sugars 1 and 2 hours after glucagon administration did not differ from control levels which suggested that little glycogen had been deposited.

E.R. was likewise studied in a relatively glycogen depleted state. A distinct negative nitrogen balance was demonstrated (Table IV) averaging 2.4 Gm. daily for the first 5 days of glucagon administration. On the sixth day, the urinary nitrogen returned to control values. The blood sugar concentration in this patient averaged 105 mg. per 100 ml. before breakfast and 153 mg. per 100 ml. 2 hours postprandially during the control periods. During the glucagon period, these averages were 104 and 145 mg. per 100 ml. respectively. The fasting plasma UFA levels averaged 1,130 μEq per liter during glucagon administration and 950 μEq per liter during control periods. In this patient, the increased urinary nitrogen was urea nitrogen.

DISCUSSION

Acute Effects.—In the light of available data, elevated plasma UFA levels may be assumed to reflect increased release of UFA from fat depots.[9] The stimuli for this release are unknown but are most likely some function of carbohydrate utilization. In those situations where glucose utilization is increased, plasma UFA levels decrease. Thus, immediately after the injection of glucagon, glycogenolysis leads to an increased blood glucose level which promotes glucose utilization. Glucagon per se may increase glucose utilization.[1, 2] Concomitant with the early increase in blood glucose, the plasma UFA levels decreased as demonstrated by the 1 hour levels in this study and as previously noted.[10] However, injection of glucagon in pharmacologic amounts resulted in a late increase in plasma UFA levels which was significantly greater than that produced by fasting alone.

This previously unnoted response does not fit with current concepts of glucagon action. There is no reason to believe that the utilization of glucose is decreased 4 to 6 hours after the injection of glucagon. In fact, although there was a prolonged elevation of blood glucose in Patients 4, 5, and 6 in response to glucagon, the UFA increase was seen at the 6 hour period.

Emotional stress has been shown to increase UFA levels.[11] This seems to be an unlikely reason for the results reported since consideration of the individual responses to saline and glucagon does not reveal a random distribution of values.

A reasonable hypothesis is that glucogon effects the release of UFA from fat depots. A direct effect of glucagon on the epididymal fat pad has been demonstrated in vitro.[12] If this is the case, 4 to 6 hours after the injection of glucagon the effect of glucagon on the release of UFA predominates over the waning effects of increased glucose utilization. That this effect of glucagon cannot be absolute was suggested by one experiment in which a meal 3 hours after injection of glucagon prevented the late rise in UFA levels.

Although the integration of this effect of glucagon in the scheme of caloric homeostasis is not clear, the following considerations are pertinent. An increase in plasma UFA levels has been associated with an increased peripheral utilization of UFA.[9] In hyperthyroidism where peripheral glucose utilization is increased, plasma UFA levels and, by inference, the utilization of UFA are likewise increased.[13] Thus, in this situation, the increased utilization of UFA apparently reflects the need for caloric substrates rather than the increased glucose utilization per se. Since glucagon has been shown to increase oxygen consumption in the rat[14] and to produce ketosis in man,[15] there are reasons for the belief that there is an increased need and utilization of calories from noncarbohydrate sources. If a similar sequence of metabolic events occurred in the fat depots 4 to 6 hours after an injection of glucagon, stimulation of UFA release would follow.

Balance Studies.—A negative nitrogen balance has been produced in man[15] and an increased urinary nitrogen excretion in the rat[16] by glucagon administration. It was suggested in the first study that glucagon exerted a direct catabolic effect since in one patient there was a negative nitrogen balance in the absence of an increased blood sugar. In the animal study the increased urinary nitrogen could not be related to the presence of liver glycogen because this effect persisted throughout a 7 day fast. After this period of time, the glycogen content of the rat liver is very low.[22]

The negative nitrogen balance which resulted from glucagon administration to A.M. was similar to that noted in patients with rheumatoid arthritis.[15] There was an immediate increase in nitrogen excretion and a prompt anabolic response occurred when glucagon was stopped. Since this subject did have cancer, it is not appropriate to term this a control study, but, except for the radiologic evidence of bone involvement, the patient was apparently normal.

The balance studies in the 2 obese women were performed in order to test the metabolic effects of glucagon in subjects relatively depleted of glycogen. The patient with stable diabetes has been shown to respond to glucagon with a lesser rise in blood sugar than does the normal subject[17] which suggested lower glycogen stores. The reducing diet should have further decreased these stores since liver glycogen is experimentally quickly depleted with inadequate calorie intake.

Thus, the negative nitrogen balance in E.R. and the suggestive changes in A.L. strengthen the evidence that glucagon is catabolic and that this is not a result of glycogenolysis and consequent gluconeogenesis. Although the time for resynthesis of glycogen is unknown in man, it is relatively slow in the rat,[18] only 50 per cent being resynthesized in 24 hours after depletion induced

by glucagon. Therefore, the frequent injections of glucagon makes it unlikely that the successive injections resulted in appreciable glycogenolysis. We cannot interpret the lack of effect of glucagon on blood glucose levels as evidence for complete depletion of glycogen, since the breakdown of a small amount of glycogen would be detected only with frequent blood sugar determinations.

The effect of glucagon in M.S. demonstrated that a catabolic effect could be obtained in the absence of measurable anterior pituitary function. Although the study was interrupted by the anorexigenic effect of the glucagon, the induced nitrogen loss was unequivocal during the last 3 days. This study and the constancy of the urinary Porter-Silber chromogen excretion in the other subjects who were receiving glucagon make it unlikely that adrenal cortical activation is responsible for the nitrogen loss. A "permissive" role of hydrocortisone in this experimental situation in the rat has been proposed.[16]

Even if these catabolic effects of glucagon represent only a pharmacologic phenomenon, the occurrence of such an effect is not explained by the only generally accepted activity of glucagon. It has been shown in the rat[14] that the injection of 1.0 mg. of glucagon resulted in an increased oxygen consumption of about 35 per cent in either the fed animal or one in the fasting condition. This dose is many times that received by our subjects, on a weight basis, but an increased oxygen consumption could be implicated in the increased nitrogen loss. The large negative nitrogen balance sustained by the patient with the sarcoma and hypoglycemia would be difficult to account for on this basis alone. Glucagon may have directly affected the tumor since experimentally antitumor effects have been found.[19] In a study with human subjects[15] ketosis was produced by glucagon concomitant with the negative nitrogen balance. This again implies either an increased caloric demand or decreased glucose utilization, and the available data do not support the latter alternative. There are thus several possible hypotheses to explain this catabolic effect of glucagon.

The continued hyperglycemia produced by glucagon in M.H. and M.S. cannot have resulted from glycogenolysis alone. Even in M.H., the 6 day nitrogen loss was 25 Gm., equal to about 150 Gm. of protein. If 65 per cent of this is potential carbohydrate, only 97 Gm. of carbohydrate was made available over the 6 day period. The patient's daily carbohydrate intake was 307 Gm., so that sugar derived from protein breakdown could account for only 5 per cent of the daily utilization. A similar phenomenon has been seen in another patient with an islet-cell carcinoma[20] and it was suggested that glucagon prevented the deposition of glycogen, which would occur in the presence of a relative excess of insulin. Such a hypothesis would fit these studies. The lack of initial or persistent hyperglycemia in the 2 obese diabetic subjects could then be explained by the low glycogen stores in the liver and the relatively decreased amounts of circulating insulin.

CONCLUSIONS AND SUMMARY

Glucagon produced an increase in plasma unesterified fatty acid (UFA) levels 4 to 6 hours after injection in 15 of 18 instances. Mechanisms for the stimulation of UFA release from the fat depots have been considered.

A negative nitrogen balance was produced by glucagon in 5 subjects. This was demonstrated to be independent of anterior pituitary activity or adrenal cortical activation.

Glucagon resulted in a persistent hyperglycemia over a 6 day period in a subject with a retroperitoneal sarcoma associated with hypoglycemia. This effect could not be due to glycogenolysis of stored liver glycogen but may have occurred as a result of inhibition of insulin induced deposition of hepatic glycogen.

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SIGNIFICANCE OF VASCULAR INJURIOUS FACTORS IN HUMAN PLASMA AS DETERMINED BY ANIMAL ASSAY

I. Influence of Nutritional Status

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The injection of fresh normal human plasma into the nonsensitized rabbit or rat induced acute injury to the capillary vascular system as evidenced by prolonged capillary dilatation, decreased vasomotor activity, increased capillary permeability, and depression of terminal arteriolar reactivity to epinephrine. Certain of these injurious effects were intensified significantly when the normal human plasma was obtained after 48 hours of starvation or several hours after fat-rich meals. This enhanced vasotoxic action of starvation, and postprandial hyperlipemic human plasma did not appear related to a specific vascular toxin but rather to a nonspecific enhancement of the unknown primary toxicity peculiar to the heterologous nature of the plasma. Although circumstantial evidence suggested the participation of lipoprotein components for the enhanced toxicity, the isolated human lipoprotein fraction (Gofman technique) did not exhibit significant vascular injurious properties. The present studies indicate that before specific clinical significance can be attributed to any vascular injurious factor detected in human plasma by animal assay, it is first necessary to demonstrate that such effects are independent of (1) the nonspecific toxicity peculiar to the heterologous character of the plasma, and (2) the nonspecific intensification of this toxicity induced by fluctuations in metabolic factors such as those which occur during alterations in nutritional status.

THE appearance during various pathologic states of humoral factors in animal and human plasma which are capable of affecting the capillary vascular system adversely has frequently been reported. Humoral factors which are injurious to the capillary system have been observed in association with essential hypertension,[1, 2] hemorrhagic and traumatic shock,[3] arthritis,[4] diabetic retinopathy,[5] radiation illness,[6] tuberculosis,[7] Cushing's syndrome,[8] hepatic decompensation, congestive heart failure, and nephrotic syndrome.[9] The demonstration of such noxious humoral factors in human plasma has generally involved the use of experimental animals, into which the plasma is injected intravenously, intraperitoneally, or intradermally, or is perfused through isolated organs. Interpretations of the physiologic derangements of illness have sometimes been based upon the results of such studies.[1-3]

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These studies, initiated at the Hemorrhagic Fever Center, Korea, during the years 1953 and 1954 and subsequently completed at the University of Maryland, Baltimore, Md., were carried out under the auspices of the Commission on Hemorrhagic Fever of the Armed Forces Epidemiological Board. Some of the earlier observations reported in this article were recorded in the Reports of the Hemorrhagic Fever Commission to the Surgeon General for the years 1953-1954 and in a brief abstract.[28]

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The original objective of this study was to search for a vascular toxin in the plasma of patients in the acute phase of epidemic hemorrhage fever which might explain the dramatic vascular disturbance encountered in this disease.[10] Initial observations suggested that such a substance might indeed be present. Closer scrutiny of the results, however, caused us to question the validity of the assay methods used regarding their capacity to detect specific toxic agents of etiologic significance in hemorrhagic fever. This, in turn, led to the discovery that the "vascular toxin" under consideration was in fact an artifact. An altered state of nutrition apparently had unexpectedly intensified the injurious vascular effects of the same general kinds observed from time to time over the years when plasma from one species of animal was injected into another species. This report presents data selected from the studies begun in Korea and terminated at the University of Maryland Medical School to demonstrate that the vascular effects of heterologous plasma can be altered to a significant degree by acute changes in the nutritional status of the plasma donor, regardless of whether he is diseased or healthy. The nature of the plasma factors involved has not yet been elucidated. The pathophysiologic significance, if any, in the human subjects from which they were derived remains open to question. Nevertheless, the results of this study help to define some of the limitations of several methods which have been devised for the detection of humoral vasoreactive substances and which employ heterologous test animals. They indicate the caution with which results obtained by such methods must be regarded as they might be intended to apply to the pathogenesis of disease states in which nutritional or metabolic disturbances occur, either primarily or incidentally.

METHODS AND MATERIALS

Assay of Human Plasma for Vascular Injurious Factors.—

1. *Rabbit:*

Vasodilator Test*.—Albino rabbits not previously exposed to human or other foreign sera, weighing 1.5 to 2.5 kilograms, were sedated with morphine sulfate† (60 to 90 mg. per kilogram). A polyethylene catheter was inserted into the marginal ear vein. The animal was then tightly wrapped in a light towel to reduce activity and the head was firmly fixed in the jaws of a condenser clamp in a position with one eye directly beneath the objective of a Leitz binocular microscope (magnification ×144). The bulbar conjunctiva, illuminated by a slit lamp, was minimally exposed by gentle traction on hemostat clamps attached to the hair of the periorbital skin. Transient conjunctival hyperemia usually followed this procedure. Animals were discarded, however, if the conjunctival hyperemia persisted. Terminal arterioles with rapid blood flow and active vasomotion were selected for study. After a control period of observation, freshly drawn human plasma was injected through the polyethylene catheter at a rate of approximately 1 ml. per 20 seconds. The total amount injected was 1 ml. per kilogram body weight of the rabbit. Alterations in diameter of the terminal arterioles were then determined by means of an ocular micrometer for periods of 15 minutes to several hours. Changes in vasomotor activity were recorded simultaneously.

Two types of responses were noted. In one, which frequently followed the injection of a wide variety of fluids including physiologic saline, a transient vasodilatation began

*Technique devised by Dr. Gordon K. Moe, Professor of Physiology, University of Syracuse, School of Medicine.

†Other agents such as barbiturates and tubocurare were found unsatisfactory because they induced persistent and marked vasodilatation of the rabbit conjunctival capillary bed.

almost immediately after injection and lasted from several seconds to approximately one minute, after which the vessel diameter rapidly returned to normal. This was considered most likely a response to the volume of injected fluid. In the other, which followed injection of some human plasmas, the vasodilatation began after a delay of 1 to 3 minutes and usually persisted 5 to 20 minutes. Such a response was often diphasic, with an initial transient vasodilation (volume effect), return to normal, and finally the delayed appearance of the prolonged vasodilation. A delayed response which lasted 5 minutes or less was considered doubtfully positive. A dilator response which lasted more than 5 minutes and was associated with a diminution of vasomotor activity was designated as a positive reaction. Occasionally an initial vasoconstrictor response occurred when human plasma was injected. This persisted several seconds to several minutes and was followed by the vasodilator phase. The delayed vasodilator response produced by human plasma differed from that produced by the intravenous injection of histamine, which in doses of the order of 5 mcg. regularly caused a more intense, immediate, and transient (1 to 2 minute) arteriolar dilatation and diminution of vasomotor activity.

This assay procedure was limited by the subjective nature of the observations and by the spontaneous fluctuations in conjunctival vascular caliber. For these reasons, after completion of the preliminary studies, all subsequent observations were conducted with a blind technique with the source of the plasma unknown to the observer. The control base-line observations of vascular caliber were followed for at least 15 minutes, and only when the injected plasma produced a definite and persistent change within the initial 3 minutes after injection of the plasma was the reaction considered positive. Fairly reproducible responses to the same plasma specimens (handled with blind technique) could then generally be obtained; of 9 tests with three negative plasma specimens, 1 positive reaction was obtained; of 10 assays with three positive plasma specimens, 2 negative assays were observed (from different plasma specimens).

Permeability Test.—The effect of human plasma on the capillary permeability of rabbit skin was assayed by intradermal inoculation into depilated abdominal skin of rabbits prepared by intravenous injections of 10 ml. of 1 per cent trypan blue solution per kilogram.[11] One tenth milliliter plasma was injected as a series of tenfold dilutions in physiologic saline ranging from undiluted plasma to dilutions up to 10^{-9}. The test sites were examined for appearance of blue discoloration 30 minutes after injection, at which time the diameter of the blue area was recorded for each dilution. Results were accepted as valid only if the animal appeared normally healthy throughout the experiment, maintained a reasonably constant skin temperature, and if saline and histamine controls produced the appropriate responses. The titer of permeability factor in plasma was defined as the highest dilution which gave a definitely positive test. When a given plasma was tested in more than one animal, the geometric mean of the titers was recorded. It is stressed that the plasma induced permeability alterations as measured here need not necessarily reflect a change in capillary endothelial permeability per se, but may represent local alterations in skin blood flow or capillary blood pressure induced by local alterations in vascular caliber. The term "permeability factor" is thus restricted to substances which produce functional increases in rabbit dermal capillary filtration of large molecules without specifying the precise mechanism.

2. *Rat:*

Mesoappendix Test.—The epinephrine reactivity of the terminal arterioles of the rat mesoappendix was titrated according to the method described by Shorr and co-workers.[3] To test the effect of human plasma on the epinephrine reactivity, 0.5 ml. human plasma was injected intravenously for each 100 grams of rat weight. The limitations of this test and the difficulties in interpretation have been documented previously.[12, 13] To mitigate these objections, only rats with a mesoappendiceal vascular threshold epinephrine reactivity that was stable during the initial 20 to 30 minutes of the control observations were employed; injections of foreign plasma were restricted to one per rat, and the source of plasma (pre- or postprandial) was withheld from the observer until completion of the assay. In addition, if a change in base-line epinephrine reactivity failed to appear within 15 minutes after

injection of the foreign plasma, the reaction was considered negative. Moreover, most assays were conducted in duplicate. Utilizing these criteria, the reproducibility of the results and the virtually consistent and marked differences obtained with the pre- and postprandial foreign plasma samples justified with some assurance, the validity of the qualitative shifts in the vascular reactivity to topical epinephrine.

Peritoneal Capillary Permeability Test.—The procedure of Courtice and Steinbeck[14] was employed. Two milliliters of human plasma per 100 grams of animal weight was injected intraperitoneally into albino rats. The residual volume of peritoneal fluid was then measured 3 hours later.

Collection and Processing of Human Plasma.—All human blood specimens were drawn into heparin coated syringes and transferred to centrifuge tubes which contained 5 drops of 1:1000 heparin per 30 ml. blood. The plasma was separated immediately by centrifugation at ambient temperature. If not tested immediately, plasma was stored in sterile vaccine bottles at 5° or -70° C.

Unless otherwise specified, all plasma used for the conjunctival vasodilator test in rabbits was inactivated by heating to 56° C. for 30 minutes* and clarified by centrifugation. For the other methods of assay, unheated plasma was used.

Fractionation of Human Plasma by Ultracentrifugation.—Three hundred milliliter samples of heparinized blood, drawn from individuals 3 hours after a breakfast of buttered toast and coffee, were immediately centrifuged. The plasma specimens were held at 5° C. until they could be subjected to ultracentrifugal fractionation by the Gofman technique.[15]† The top, middle, and bottom fractions thus obtained were dialyzed at 5° C. for 2 hours with mechanical stirring against a 1 per cent gelatin solution in physiologic saline (to avoid plasma dilution), and assayed for vascular injurious activity. In three trials, the bottom plasma layer was subfractionated by increasing the specific gracity to d = 1.121 with sodium nitrate and recentrifuging. The top, middle, and bottom layers resulting from this procedure were similarly dialyzed and assayed for vascular injurious factors. All fractionated samples were kept at 5° C. and tested within 48 hours.

RESULTS

1. *Humoral Vascular Injurious Factors in Patients With Hemorrhagic Fever and Other Febrile Illnesses.*—The intravenous injection of 1.0 ml. per kilogram of heat inactivated freshly drawn plasma from patients acutely ill with hemorrhagic fever into rabbits which had undergone morphinization usually induced a conjunctival reaction characterized by a delayed (1 to 3 minute) but persistent (5 to 20 minute) dilatation and diminution of vasomotor activity of the terminal arterioles. Control plasma specimens drawn from normal subjects, from patients with other febrile illnesses, or from convalescent patients with hemorrhagic fever usually yielded negative vascular responses (Table I).

In evaluating the significance of these observations, it became apparent that nutritional status must be considered since most patients during the acute phase of hemorrhagic fever were in a state of starvation. They had usually

*Preliminary experiments indicated that fresh human plasma injected intravenously in repeated doses of 2 to 3 ml., whether from ill subjects or normal control subjects, caused progressive deterioration of 1.5 to 2.0 kilogram rabbits, with dilatation, hemorrhages, and edema of the conjunctival capillary bed, gross hematuria, retroperitoneal edema, terminal hypotension and death in convulsions. At the suggestion of Dr. Colin M. McLoed, Professor of Research Medicine, University of Pennsylvania, plasma was heat inactivated. Repeated doses of 2 ml of such inactivated plasma from normal subjects were found to be well tolerated by test animals which weighed 2 kilograms.

†The ultracentrifugal fractionation technique was performed by personnel of the Department of Virus and Rickettsial Diseases, Walter Reed Army Institute of Research, Washington D. C.

TABLE I. VASODILATOR REACTION IN RABBITS IN RESPONSE TO INJECTION OF PLASMA FROM PATIENTS WITH AND WITHOUT HEMORRHAGIC FEVER

DIAGNOSIS	DAY OF DISEASE	NO. OF PATIENTS	NO. WITH POSITIVE VASODILATOR REACTION	
Hemorrhagic fever	1	2	0	Acute phase of illness
Hemorrhagic fever	2	3	2	
Hemorrhagic fever	3	15	15	
Hemorrhagic fever	4	9	8	
Hemorrhagic fever	5	2	2	
Hemorrhagic fever	7	2	1	
Hemorrhagic fever	12	1	0	
Hemorrhagic fever	15	1	0	
Hemorrhagic fever	21	1	0	
Hemorrhagic fever	23	2	0	
Hemorrhagic fever	28	1	0	
Hemorrhagic fever	30	1	0	
Normal	—	9	1	
Pneumonia	2-5	5	0	
Upper respiratory infection	2-5	2	0	
Malaria	2-5	2	0	
Infectious mononucleosis	2-5	2	1	
Fever, unknown origin	2-5	2	2	

undergone several days of intense anorexia, often associated with protracted vomiting and with acetonuria. Studies were therefore performed to determine the influence that intravenous infusion of glucose solutions to, or resumption of food intake by, the plasma donor would have on plasma vasodilator activity. Intravenous infusions of one to two liters of 10 per cent glucose solutions or the oral intake of comparable quantities of carbohydrate was usually followed within 24 hours by the disappearance of the plasma vasodilator activity *despite an unaltered clinical picture*. Furthermore, the disappearance of plasma vasodilator activity was found to parallel the decline in acetonuria (Table II).

2. *Effect of Nutritional Status on Vascular Injurious Reactions Induced by Human Plasma From Normal Subjects.*—Since the previous findings suggested that nutritional status, rather than disease per se, may determine the

TABLE II. CORRELATION OF NUTRITIONAL STATUS AND ACETONURIA WITH VASODILATOR EFFECT OF HEMORRHAGIC FEVER PLASMA

SUBJECT	ANOREXIA ON ADMISSION		VASODILATOR EFFECT OF PLASMA*		
	DEGREE	DURATION (HR.)	ON ADMISSION	24 HR. AFTER	48 HR. AFTER
1	Severe	48	+(2+)	0(±)	
2	Severe	92	+(3+)	+(1+)	0(0)
3	Severe	46	+(3+)	±(2+)	
4	Severe	60	+(3+)	+(2+)	
5	Severe	48	+(1+)	0(0)	
6	Severe	72	+(3+)		+(1+)
7	Severe	36	+(±)	0(0)	
8	Severe	24	0(0)	+(3+)	
9	Moderate	48	+(1+)	0(0)	
10	Mild	68	±(1+)		
11	Mild	48	0(0)	0(0)	
12	Mild	24	0(0)		

*Figures in parentheses represent degree of acetonuria.

vascular action of human plasma in the experimental animal, the effect of altering the nutritional status of normal subjects was tested. Accordingly, paired samples of normal plasma were assayed; one sample was obtained 12 to 18 hours postprandially and the other sample 2 to 4 hours postprandially or after 48 hours of starvation. The results are indicated in Table III. Conjunctival vasodilator activity for the rabbit, consistently absent in the 12 to 18 hour fasting plasma samples, generally appeared during the postprandial and starvation states. The permeability factor titer was also found to increase significantly in those plasma specimens drawn postprandially or after starvation when compared with specimens from the same subjects 12 to 18 hours after fasting. The conjunctival vasodilator activity and increase in permeability factor titer in the postprandial plasma specimens developed only after large fat-rich meals consisting of liberal quantities of fried meat, fried potatoes, butter, and milk or cream.

TABLE III. VASODILATOR AND PERMEABILITY FACTORS IN PLASMA OF NORMAL HUMAN SUBJECTS DURING POSTPRANDIAL, FASTING, AND STARVATION STATES

	PERMEABILITY TITERS* (HR. AFTER EATING)			VASODILATOR RESPONSE (HR. AFTER EATING)		
SUBJECT	2-4	12-18	48	2-4	12-18	48†
1	7	2		+	−	
2	7	3		+	−	
3	6	4		−	−	
4	6	4		+	−	
5	7	4		+	−	
6‡	8	8		±	−	
7				+	−	
8				+	−	
9		2	7		−	+(3+)
10		1	2		−	±(3+)
11		3	5		−	+(4+)
12		2	5		−	+(3+)
13		2	4		−	+(4+)
14		4	2		−	+(3+)
15		5	3		−	±(3+)
16		3	7		−	+(3+)

*Titer expressed as log 10 of the reciprocal of highest dilution which gave a positive test.
†Figures in parenthesis represent degree of acetonuria.
‡Subject did not eat all of fat-rich meal.

The results of the preceding experiments were based upon comparisons of paired plasma samples drawn from the same subjects at intervals as far apart as 36 hours (i.e., the 12 and 48 hour fasting specimens). Since both plasma samples were assayed simultaneously, the possibility was explored that loss of activity occurred during storage of the initial plasma samples at 5° C., thus producing an apparent rather than a real increase in the vascular injurious effect of the second plasma sample. Consequently, five individual plasma specimens stored at 5° C. were retested at varying intervals. No significant decline in properties injurious to the vascular system were detected during the initial 48 hours of storage.

Since alterations in nutritional status produced significant differences in the effects of human plasma on the conjunctival and cutaneous capillary vessels

of the rabbit, studies were undertaken to determine if such reactions were restricted to the rabbit. Paired fasting and postprandial specimens of human plasma from eight healthy subjects (not employed in the previous studies) were injected intravenously into anesthetized albino rats. In the dose employed, the 12 to 18 hour fasting plasma samples uniformly produced no detectable effect upon the threshhold epinephrine reactivity of the terminal arterioles of the rat mesoappendix. In contrast, the majority of plasma samples drawn 2 to 4 hours postprandially from the same subjects elicited definite vasodepressor responses as indicated by depression of the threshold responsiveness of the terminal arterioles to topical epinephrine, Table IV. Large fat-rich meals, similar to those described for the rabbit vasodilator and permeability factor assays were employed for these experiments. Comparable alterations in nutritional state, however, did not enhance appreciably the ability of whole human plasma to increase the peritoneal capillary permeability of the rat.

TABLE IV. EFFECT OF PAIRED FASTING AND POSTPRANDIAL PLASMA SPECIMENS FROM NORMAL HUMAN SUBJECTS ON THE THRESHOLD EPINEPHRINE REACTIVITY OF TERMINAL ARTERIOLES OF THE RAT MESOAPPENDIX

NUTRITIONAL STATUS	NO. OF SUBJECTS	THRESHOLD EPINEPHRINE REACTIVITY		
		NO. SPECIMENS* INDUCING DEPRESSED REACTIVITY	DURATION OF DEPRESSED REACTIVITY (MIN.)	MAGNITUDE OF REACTIVITY DEPRESSION
Fasting (10-18 hr.)	8	0	—	—
Postprandial† (2-3 hr.)	8	7‡	15-80 (Average 35)	Two- to twenty-fold (Average 6)

*Standard plasma dose of 0.5 ml. per 80 to 125 grams was given intravenously to test rats, usually in duplicate.

†In the 3 instances tested, ether extraction of plasma at 0° C. abolished the vasodepressor action of plasma.

‡The single plasma which failed to depress epinephrine reactivity showed only minimal postprandial lipemia.

TABLE V. EFFECTS OF ULTRACENTRIFUGED FRACTIONS* OF HUMAN PLASMA ON PERMEABILITY OF CAPILLARY VESSELS OF RABBIT AND RAT

PLASMA SPECIMEN	PERMEABILITY TITER†							RAT PERITONEAL EXUDATION‡							
	1	2	3	4	5	6	7	1	2	3	4	5	6	7	8
Whole		1	2	5	2	2	2		2	6	31	40		18	35
Top	0	0	0	0	0	0	1		4	2	2	3		3	10
Middle	3	0	2	2	1	2	2		3	2	6	33		5	18
Bottom	4	0	3	4	2	2	5		12	18	26	58		27	38

*Fractions prepared by Gofman technique.

†Titer expressed as the highest dilution of plasma (negative exponent of 10) giving a positive test in the rabbit skin after preparation with trypan blue.

‡Milliliter per kilogram of fluid removed from the peritoneal cavity 3 hours after the intraperitoneal injection into rats of 20 ml. plasma fraction per kilogram (average of groups of 3 rats).

3. *Vascular Injurious Factors in Fractions of Normal Human Plasma Prepared by Ultracentrifugation.*—The effect of heterologous capillary permeability of human plasma fractions prepared by ultracentrifugation of eight plasma samples is indicated in Table V. The isolated lipoprotein-rich fraction (top layer) prepared according to the Gofman technique was relatively inert. The

factors which were significant in enhancing capillary permeability were consistently associated with the sedimented fractions. Similarly, positive rabbit vasodilator responses were obtained with six of the seven bottom fractions tested and with but two of the sixteen top and middle fractions. However, no vasodepressor (or vasoexcitor) activity was elicited in the rat mesoappendix assay system by any of the whole or fractionated plasma specimens. Attempts to subfractionate the sedimented plasma layers by increasing the specific gravity and recentrifuging generally resulted in diminution or loss of all vascular injurious activity.

4. *Vascular Injurious Activity in Relation to the Source of the Plasma.*—The enhancement of the vascular injurious effects of human plasma for heterologous species by alterations in nutritional status did not appear to be peculiar to the human. Whereas the intravenous injection of normal rabbit plasma (0.5 ml. per 100 grams rat weight) failed to alter the threshold epinephrine reactivity of the terminal arterioles of the rat mesoappendix, vasodepressor effects were consistently obtained with plasma drawn from the same rabbits 2 to 4 hours after they were fed two raw eggs by tube (Table VI). These vasodepressor effects could not be correlated with alterations in neutral fat, phospholipid, or cholesterol content of the rabbit plasma, or with alterations in the ratio of these substances to each other.*

TABLE VI. EFFECT OF PLASMA FROM NORMAL AND EGG-FED RABBITS ON THE THRESHOLD EPINEPHRINE REACTIVITY OF TERMINAL ARTERIOLES OF THE RAT MESOAPPENDIX

NUTRITIONAL STATUS	NO. OF RABBITS	NO. SPECIMENS DEPRESSING EPINEPHRINE REACTIVITY	DURATION OF DEPRESSED REACTIVITY (MIN.)	MAGNITUDE OF REACTIVITY DEPRESSION
Normal*	10	0	—	—
Egg fed†	11	10	27-195 (Average 72)	3-20× (Average 8×)

*All normal specimens were drawn from rabbits subsequently fed eggs.

†Plasma drawn 2 to 4 hours after rabbits, which weighed 1.5 to 2.0 kilograms, were fed two eggs by tube.

In alternate groups of rabbits (3 in each series), neither did the feeding of cottonseed oil or butter, emulsified with gum acacia, nor acute starvation for periods of up to 7 days, result in vasodepressor plasma activity. It may be significant that acetonuria failed to appear in the latter group of animals.†

The injection of fresh normal *homologous* heparinized plasma was not observed to induce significant vascular alterations in the rabbit or rat. Variations in nutritional status did not affect these results. Thus the intravenous injection of fresh unheated rabbit plasma obtained 2 to 4 hours after egg feeding exerted no effect upon the caliber or vasomotor activity of the conjunctival capillary bed of homologous species, even in doses of 5 ml. per kilogram. Ra

*Analysis performed by the Department of Biochemistry, 406th Medical General Laboratory, Tokyo, Japan, under the direction of Lieutenant Colonel Jeffries.

†That the initial cardiac puncture and bleeding of 2 ml. per kilogram necessary to obtain the control plasma samples did not per se induce the subsequent vasodepressor plasma effect of the rabbits which were fed eggs was indicated by the negative results obtained with the rabbits which were fed cottonseed oil and butter. However, the extent to which the trauma of the initial blood sampling might have contributed to the vasodepressor effects was not evaluated.

plasma drawn 2 to 4 hours after rats were tube fed 3 ml. egg yolk per 100 grams weight or after 5 to 7 days of acute starvation exerted no vasodepressor effect upon the threshold epinephrine reactivity of the terminal mesoappendiceal arterioles of the homologous species.

DISCUSSION

The property of plasma or serum obtained from normal animals or humans to induce circulatory collapse and death when injected into heterologous species has long been established.[16] Moreover, the injection of such "normal" foreign plasma or serum may induce acute and severe capillary injury as evidenced by the appearance of petechial hemorrhages, capillary dilatation, and interstitial edema at postmortem examination.[4, 5, 17] The precise mechanism for these vascular injurious reactions has not been defined. Various investigators have alternatively postulated that the vasotoxic effects of foreign plasma or sera are dependent upon the ability of such material to induce intravascular erythrocyte agglutination and/or hemolysis,[18] affect the neurogenic control of the vascular tree,[19] predispose to intravascular clotting,[20-23] or activate toxic humoral factors such as anaphylotoxin.[16]

During the past decade, a number of reports have appeared concerning the occurrence of factors injurious to the vascular system in human plasma or serum during various disease states. The majority of these investigations were conducted by injection of human plasma into one or more of the common laboratory animals. Thus in relation to human sera as assayed in the rat, rabbit, or guinea pig, factors injurious to the vascular system have been reported during various infectious illnesses,[4, 7] hepatic decompensation, congestive heart failure and the nephrotic syndrome,[9] arthritis,[4] Cushing's syndrome,[8] essential hypertension,[1-3] and diabetic retinopathy.[5]

The significance of these findings in relation to each disease state is uncertain. Zweifach and Metz[24] have emphasized the inability to interpret the responses elicited by human plasma in the rat mesoappendix assay system in terms of specific systemic physiologic derangements. Lovell and associates[4, 25] have stressed the apparent nonspecificity of the capillary vascular injurious effect of human serum in the guinea pig skin. However, in most of the aforementioned studies, no attempt has been made to determine whether such properties injurious to the vascular system of human serum during illness represent the elaboration of specific vasotoxic factors or merely a *nonspecific intensification* of the intrinsic toxicity peculiar to the heterologous nature of the serum. Such a distinction would be of fundamental importance, since the elaboration of specific capillary injurious factors would be highly significant in the pathogenesis of disease, whereas toxic effects referable primarily to the heterologous nature of the plasma would require a different interpretation as to possible direct clinical significance.

During various pathologic states, nonspecific alterations in nutritional status, which result in marked changes in plasma composition, may occur. Diminished food absorption caused by anorexia, vomiting, or diarrhea, dehydration

with hemoconcentration, accelerated tissue destruction, and exhaustion of glycogen reserves with increased lipid mobilization and breakdown are some of the general metabolic changes that frequently develop. Even in the normal individual, significant alterations in plasma composition may be induced simply by the ingestion of a large meal. Despite the well-recognized occurrence of such nonspecific metabolic alterations, the influence of these factors on the vascular injurious properties of plasma or serum has received scant attention. Novy and De Kruif[26] reported that diet appeared to affect the ability of rabbit serum to be rendered toxic for guinea pigs by the addition of agar. Lake and co-workers[5] concluded that the ability of heterologous plasma to increase peritoneal capillary permeability depended upon the lipoprotein fraction, and that this effect (at least with rabbit serum) could be enhanced significantly by feeding substances which raised the plasma lipoprotein levels.

The data obtained during the present investigation indicate that freshly drawn normal human plasma is capable of acutely injuring the small blood vessels of such heterologous species as the rabbit and rat and that this injurious effect may be enhanced significantly merely by altering the nutritional status of the donor. The samples of hyperlipemic human plasma obtained 2 to 4 hours after the ingestion of fat-rich meals and the samples of watery clear plasma obtained after 48 hours of starvation fairly consistently invoked more intense capillary injurious effects when injected into the experimental animals than did the specimens obtained from the same donors after a 12 to 18 hour fast. These capillary injurious effects were evidenced by the appearance of prolonged capillary dilatation, decreased vasomotor activity, increased capillary permeability (this term is employed as a functional one and does not necessarily specify increased endothelial permeability per se), or depression of the reactivity of terminal arterioles to topically applied epinephrine.

That this relation of nutritional status to intensity of the capillary injurious properties of plasma for heterologous species is not peculiar to the human plasma donors was suggested by the enhanced ability of rabbit plasma obtained after egg feeding to depress the epinephrine reactivity of the terminal arterioles of the rat mesoappendix. However, since feeding of raw egg white can induce toxic effects and even death in rabbits[27] the mechanism for this increase in postprandial plasma vasotoxicity may differ from that in the human. Furthermore, species differences in intermediary metabolism may modify the role that nutritional status assumes in influencing plasma vascular injurious activity for heterologous species. Thus after 48 hours of starvation, 3 to 4 plus acetonuria developed in each of eight normal volunteers, whose plasma samples at this time consistently exhibited an intensification of the heterologous vascular injurious effects. In contrast, even after 7 days of starvation, acetonuria failed to appear in each of three test rabbits and plasma samples drawn at this time showed no detectable mesoappendiceal vasodepressor effects upon intravenous injection into the rat.

Since enhancement of the acute vascular injurious effects of human plasma (1) occurred after fat-rich meals and after acute starvation, (2) paralleled the

appearance of acetonuria during starvation, and (3) decreased rapidly after infusions of glucose during the starvation state in hemorrhagic fever, it seemed that certain of the plasma lipoproteins might be responsible. However, the isolated lipoprotein-rich fractions prepared by ultracentrifugation according to the Gofman technique caused no significant vascular injurious reactions as determined by any of the assay procedures utilized in these studies. Nevertheless, the more critical data concerning the effects of such isolated lipoprotein fractions acting *in conjunction* with other components of the whole plasma have yet to be determined. Indeed, later experiments, to be described in a subsequent paper, suggest that certain human lipoprotein fractions do enhance the vascular injurious effects of whole human plasma for the rabbit by a complex series of reactions which are interrelated with basic immunologic mechanisms.

In contrast to the effect of fresh heterologous plasma, normal rabbit or rat plasma consistently failed to exhibit vascular injurious activity when injected into the homologous species. Despite drastic changes in nutritional status, homologous plasma from these animals, even in doses two- to fivefold greater than those which induce vascular reactions in heterologous animals, produced no detectable capillary changes. Thus the humoral factors associated with altered nutritional status appear to *intensify* the intrinsic capillary injurious activity peculiar to the foreign plasma reaction rather than induce a new type of vascular injurious effect.

Many of the previous assay methods for detecting vascular injurious factors in human plasma during illness have employed normal human plasma as the control. As a consequence, the existence of "specific" capillary injurious factors was usually accepted if the injection of plasma drawn from patients with a specific disease produced vascular alterations in the test animal in a lower dosage range than did "normal" plasma. The inferences of the role of VDM in the plasma of subjects with such diseases as hemorrhagic and traumatic shock, Cushing's syndrome, decompensated hepatic cirrhosis, essential hypertension, the nephrotic syndrome, and congestive heart failure[1-3, 8, 9] may be cited as one such example. The findings of this present investigation indicate, however, that before clinical significance can be attributed to any vascular injurious factors detected in human plasma by animal assay, it is first necessary to demonstrate that these injurious effects are independent of (1) the nonspecific toxicity peculiar to the heterologous character of the plasma and (2) the nonspecific enhancement of this foreign plasma toxicity produced by variations in nutritional status. Furthermore during illness, the possibility that incidental fluctuations of metabolic factors other than those affected by nutrition may similarly act by intensifying the foreign plasma toxicity must also be excluded.

SUMMARY

The injection of fresh normal human plasma into the rabbit or rat induces acute injurious effects to the capillary vascular system. The injurious effects are characterized by prolonged capillary dilatation, decreased vasomotor activity, increased capillary permeability, and depression of the reactivity of terminal arterioles to epinephrine. Certain of these vascular injurious effects may be

enhanced significantly if the human plasma is obtained after 48 hours of starvation or after large fat-rich meals. Such intensification of the vasotoxic action by starvation or postprandial hyperlipemic human plasma does not appear related to the appearance of a specific vascular toxin but rather to a nonspecific enhancement of the unknown primary toxicity peculiar to the heterologous nature of the plasma. It does not necessarily indicate that similar, or even any, kind of action was being induced in the subject from which the specimen was taken. Although circumstantial evidence suggested that lipoprotein components might be responsible for the enhancement of the vascular toxicity of starvation and hyperlipemic human plasma for the rabbit and rat, the isolated human lipoproteins prepared by the Gofman technique did not exhibit any significant acute vascular injurious properties.

The influence of certain disease states in intensifying the capillary injurious effects of human (or animal) plasma when assayed in heterologous species has at times led investigators to conclude that specific humoral factors injurious to the vascular system are concerned in the pathogenesis of these illnesses. The data obtained during this present study indicate the necessity for critical evaluation of all studies relating humoral vasotoxic factors to the pathogenesis of illness. The findings in this study clearly indicate that before specific clinical significance can be attributed to any vascular injurious factors detected in human plasma by animal assay, it is first necessary to demonstrate that these injurious effects are independent of (1) the nonspecific toxicity peculiar to the heterologous character of the plasma, and (2) the nonspecific intensification of this plasma toxicity induced by fluctuations of metabolic factors, including those that occur during alterations in nutritional status.

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FURTHER STUDIES ON THE HETEROZYGOUS CARRIER IN GALACTOSEMIA

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In the present report the measurement of galactose-1-phosphate uridyl transferase among fourteen galactosemic families is described. The enzyme has been assayed using four different methods, two of which are based upon uridine diphosphoglucose (UDPG) consumption and two upon Warburg measurements of oxygen uptake.

In the course of these studies, a consistent lag period was noted after the addition of galactose-1-phosphate. It was shown that the concentrations of phosphoglucomutase, glucose-1,6-diphosphate, uridine diphosphoglucose, galactose-1-phosphate, and epimerase were not the limiting factors in this system. On the other hand, the lag could be corrected by supplementation with glucose-6-phosphate dehydrogenase.

GALACTOSEMIA is a hereditary disorder of carbohydrate metabolism characterized by vomiting, diarrhea, jaundice, poor weight gain, and malnutrition during early infancy. Mental retardation, cataracts, and cirrhosis of the liver are sometimes seen in this condition. Clinically, the diagnosis is usually established on the basis of an abnormal galactose tolerance curve and the presence of galactose in the urine. In 1956, Schwarz and co-workers[1] showed that galactose-1-phosphate accumulates in the erythrocytes of children with galactosemia, if they receive galactose or milk. In the same year, Kalckar and collaborators[2] demonstrated a deficiency of galactose-1-phosphate uridyl transferase in the erythrocytes of these children. This indicated that the site of the metabolic block in this disease was at the step of:

$$\text{galactose-1-PO}_4 + \text{UDP-glucose} \rightleftharpoons \text{UDP-galactose} + \text{glucose-1-PO}_4$$

The frequent occurrence of this condition in siblings and among the offspring of consanguineous matings, together with its equal distribution in both sexes, suggests that galactosemia is probably transmitted by a single autosomal recessive gene.[3] In this situation, the heterozygous carriers for the abnormal gene will usually be free of clinical symptoms. Careful biochemical studies, however, might reveal minor departures from the norm.

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In 1955, Holzel and Komrower[4] undertook galactose tolerance tests among parents, siblings, and near relatives of 6 families with many cases of galactosemia. They found the response to be abnormal in a number of the relatives tested, but this was not a consistent finding among all the individuals who should be heterozygotes. Furthermore, in a majority of families, only one of the parents showed an elevation of the galactose tolerance test. Recently, Donnell and associates[5] have repeated these studies in 29 members of 13 families including 21 children with galactosemia. A reduced tolerance to galactose loading in both mothers and fathers of affected children was noted in many instances, but there was considerable overlap between the range of values in normal individuals and heterozygotes.

Huang and co-workers[6] measured hexose phosphate levels in the erythrocytes after a galactose load. Although a three- to fivefold increase above fasting levels of this fraction of phosphate esters was observed in the galactosemic children, there was no such increase among heterozygotes as compared with normal control subjects.

In 1958, Hsia and coauthors[7] reported a decrease of galactose-1-phosphate uridyl transferase among parents of galactosemic children using the uridine diphosphoglucose consumption test described by Anderson and associates.[8] Although the enzyme levels were significantly lower among the heterozygotes, it was found that considerable overlap occurred when compared with those of normal control subjects. Subsequently, a decrease of galactose-1-phosphate uridyl transferase was noted by Bretthauer and collaborators.[9] The assay procedure was modified using a higher concentration of uridine diphosphoglucose, a higher ratio of galactose-1-phosphate (Gal-1-P) to uridine diphosphoglucose, less hemolyzate, and a shorter incubation period to maintain maximum enzyme activity, and the overlap was greatly reduced. Finally, Kirkman and Bynum[10] established a direct manometric method for the assay of transferase. This system includes the use of supplementary phosphoglucomutase and glucose-1,6-diphosphate to facilitate the removal of glucose-1-phosphate (G-1-P). A good separation between heterozygotes and normal control subjects can be obtained using this technique.

The present communication is a report on galactose-1-phosphate uridyl transferase activity measurements in members of 14 galactosemic families. Four different assay methods were used and several other observations regarding the maintenance of consistent kinetics in this enzyme system are described.

METHODS

The genetic data on the families are summarized in Fig. 1. In each instance, the diagnosis of galactosemia was established by a typical clinical history of failure to thrive and feeding difficulties since birth, jaundice and an enlarged liver, a reducing substance in the urine after the first week of life, and albuminuria. The reducing substance in the urine was demonstrated to be galactose either by the failure to ferment with yeast, positive mucic acid test, osazone crystal formation, or by paper chromatography.

Preparation of Hemolyzate.—Blood was collected in a heparinized tube and spun down in an International centrifuge for 20 minutes at 600 g. The plasma was aspirated

off by means of a capillary pipette and care was taken to remove the white cells completely. The red cells were then washed three times with an equal volume of cold 0.9 per cent solution of sodium chloride. The washed packed red blood cells were then hemolyzed by the addition of an equal volume of distilled water. The hemolyzate was rapidly frozen and thawed three times to ensure complete hemolysis of the red blood cells.

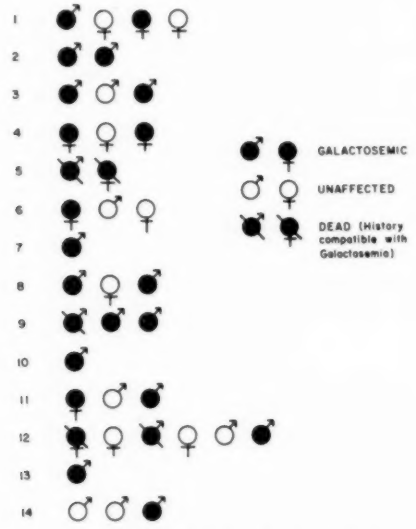

Fig. 1.—Summary of family histories in 14 galactosemic families.

Estimation of Hemoglobin.[11]—The amount of hemoglobin in the hemolyzate was determined as follows: 20 μl of the final hemolyzate (a microvolumetric pipette was employed) was added to 5 ml. ammonia water (4 ml. concentrated ammonium hydroxide, specific gravity 0.88 made up to 1 L. with distilled water). The solution was then mixed and read in a Beckman spectrophotometer at 540 mμ. The reading on the logarithmic scale (O. D.) multiplied by 0.282 gives the concentration of hemoglobin in grams per milliliter of hemolyzate.

*Assay of Galactose-1-Phosphate Uridyl Transferase.**—

Method A: The UDPG consumption test as described by Anderson and collaborators[8] was used.

Twenty-five hundredths milliliter of hemolyzate was added to 54.5 μM tris buffer, 0.375 μM galactose-1-phosphate, 0.14 μM UDPG, and made up to a final volume of 0.6 ml. The mixture was incubated for 30 minutes at 37° C. For each sample of blood, a control was run in which no galactose-1-phosphate was added. At the termination of the incubation period, 1 ml. of distilled water was rapidly added, and the mixture was then heated at 100° C. for 2 minutes with rapid and constant stirring with a glass rod. The sample was then cooled and centrifuged. The amount of residual UDPG in the heat inactivated supernatant was determined by use of uridine diphosphoglucose dehydrogenase in the presence of diphosphopyridine nucleotide (DPN). The increase of optical density at 340 mμ brought about by the reduction of DPN to DPNH was measured with a Beckman DU spectrophotometer until the optical density reached a maximum, which usually takes about 20 to 30 minutes. Beckman cuvettes of 1 cm. light path and 1.5 ml. capacity were used. Each cuvette contained 0.3 ml. supernatant, 0.15 μM glycine buffer (pH 8.7), 6 μM cysteine (prepared freshly before use and adjusted to a pH between 7 and 8 with 1N NaOH), 1μM DPN (pH 7 .

*Unless otherwise stated, all of the special reagents were obtained from Sigma Chemical Company, St. Louis, Mo.

and 0.1 ml. of UDPG dehydrogenase* (800 units per milliliter) in a final volume of 1 ml. The difference between the changes of O.D. at 340 mμ of the blank and sample constitutes the basis for the calculations of Gal-1-P uridyl transferase.

One unit was defined as that amount of enzyme which would convert one micromole of UDPG to UDPGal per hour. Units of Gal-1-P uridyl transferase per milliliter of hemolyzate are obtained as follows:

$$\frac{(\Delta 1' - \Delta 2') \times V \times t}{f \times v \times E}$$

$\Delta 1'$ = difference between initial and final reading at 340 mμ (control).† $\Delta 2'$ = difference between initial and final reading at 340 mμ (sample).† V = 1.6 ml. (total volume of incubation mixture at end of incubation). f = 12. (By the method used, the conversion of one micromole of UDPG in 1 ml. of mixture would correspond to a Δ O.D. of 0.120 at 340 mμ.) v = 0.3 ml. (volume from incubation mixture used for assay of UDPG). E = 0.25 ml. (volume of hemolyzate used in incubation mixture). t = 2 (factor to bring incubation period to 1 hour).

For each hemolyzate, the diphosphoglucose pyrophosphorylase was assayed to assure that the blood sample and the hemolyzate had been properly handled. The Gal-1-P uridyl transferase was expressed as units per gram of hemoglobin.

Method B: The UDPG consumption test as described by Bretthauer and associates[9] was used.

After the reagents were brought to 37° C. the following incubation conditions were employed:

Flask 1.—Flask 1 contained 0.20 μM UDPG, 0.80 μM Gal-1-P, 0.10 ml. 1M glycine buffer (pH 8.7), and 0.20 ml. hemolyzate in a final volume of 0.44 ml.

Flask 2.—Flask 2 contained 0.20 μM UDPG, 0.10 ml. 1M glycine buffer (pH 8.7), and 0.20 ml. hemolyzate, and was made up to a total volume of 0.44 ml. Incubation time was 10 minutes at 37° C. The reaction was stopped by placing the reaction flasks in a boiling water bath for 2 minutes and subsequently into ice water. A filtrate was then obtained by centrifugation and assayed for residual amounts of UDPG.

The filtrate from each flask was assayed in the following manner: 0.06 ml. of filtrate, 0.6 μM DPN (pH 7.4), 1.68 ml. 0.1M glycine buffer (pH 8.7), and 0.06 ml. UDPG dehydrogenase (800 units per milliliter), made to a total volume of 3.0 ml.

After the solution was mixed, it was then read in a Beckman DU Spectrophotometer at 340 mμ every 30 seconds for the first 2 minutes after the UDPG dehydrogenase was added. The optical density was then recorded at the end of the reaction, which takes approximately 90 minutes. (By increasing the concentration of UDPG dehydrogenase fivefold, this reaction can be made to end in 10 minutes. However, the total change in optical density remained the same.) The change in optical density was then determined by extrapolating back to the optical density at zero time and subtracting this value from the final value.

The results are expressed as micromoles UDPG consumed per hour per gram hemoglobin. The formula used is the same as that in method A. In this method, V = 0.44, t = 6, f = 4. (In this method, the conversion of 1 μM of UDPG in the 3 ml. of mixture, would correspond to a Δ O.D. 340 of 0.040.) v = 0.06, and E = 0.20.

Method C: The oxygen uptake method as described by Kirkman and Bynum[10] was employed.

Incubation conditions for O_2 uptake were as follows: 6 ml. single side arm flasks were used, each containing 21 μl 0.1M $MgCl_2$, 14 μl 1.5M nicotinamide, 7 μl 0.04M triphosphopyridine nucleotide (TPN), 35 μl 0.06M UDPG, 0.5M potassium phosphate buffer (pH 7.4), 1.4 μl glucose-1,6-diphosphate (1.0 μM per milliliter), 30 μl 0.2M cysteine (neutralized), 5 μl phosphoglucomutase (concentration sufficient to give a rate of 5 μM per hour), 20 μl methylene blue, 300 μl hemolyzate, and 50 μl 10 per cent KOH in the center well.

*Prepared through Step 5 according to the method of Strominger and co-workers[11] (Sigma Chemical Company).

†Both of the readings are corrected for nonspecific density changes (O.D. at 400 mμ) at 2½ minutes and at termination of assay.

In addition to this mixture, two Warburg flasks contained 50 μl 0.1M Gal-1-P, and a third flask, which served as a blank and control, contained 50 μl 0.02M G-1-P. Each flask with a total fluid volume of 1,000 μl was then placed in a 38° C. constant temperature bath with a shaker. Air was the gas phase in this system. The systems were then closed after a period of 10 minutes and readings were taken every 5 minutes. After 15 minutes, the galactose-1-phosphate was tipped in; 60 minutes later, the glucose-1-phosphate in the third Warburg flask side arm was tipped in and readings were taken until the O_2 uptake was negligible. The results were expressed as microliters O_2 uptake per hour per 0.3 ml. whole hemolyzate.

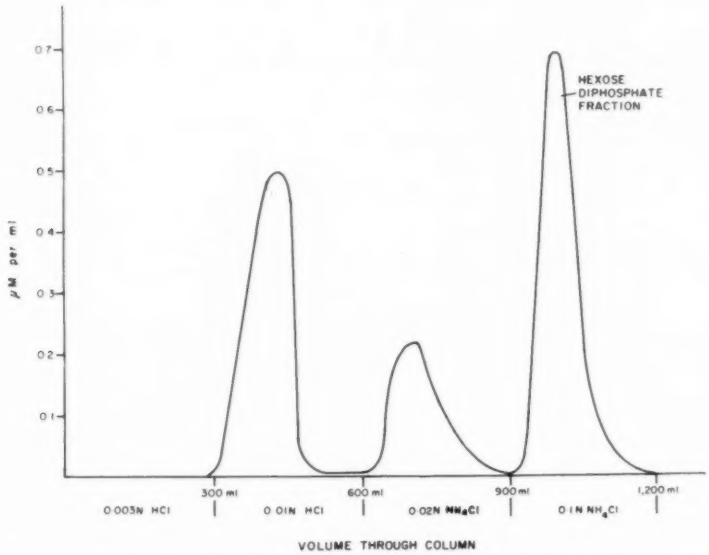

Fig. 2.—Diagram illustrating the elution of the hexose diphosphate fraction on Dowex-1-chloride column.

Method D: The procedure and reagents employed were the same as described except phosphoglucomutase and glucose-1,6-diphosphate were deleted from the flasks. However, 35 μl 0.1M UDPG was now employed. The results were expressed as microliters O_2 uptake per hour per 0.3 ml. whole hemolyzate.

Preparation of Glucose-1,6-Diphosphate.[12]—

1. *Preparation of blood:* Blood was collected in heparin (0.2 mg. per milliliter blood), cooled, and centrifuged for 20 minutes at 3,000 g. The plasma and the top 5 per cent of the cells were aspirated off. The packed red blood cells were then washed in two volumes of 0.15N NaCl, centrifuged, and the saline and surface layer of cells were drawn off and discarded. This was repeated again. The packed red blood cells were then poured with efficient stirring into two volumes of 10 per cent trichloroacetic acid and centrifuged. The supernatant was removed and stored in the cold and the precipitate was then extracted again with two volumes of 5 per cent trichloroacetic acid and centrifuged. The supernatant was removed and added to the first supernatant solution. Up until this point all manipulations were maintained at approximately 5° C.

The trichloroacetic acid was then removed from the supernatant solutions by extraction with two volumes of ether. This procedure was repeated four times. The residual ether then was removed by evaporation with a stream of nitrogen.

The solution, which had been freed of ether and trichloroacetic acid was now passed through a column of Dowex 50-X 8H (60 to 80 wet mesh) at a flow rate of approximately 5 ml. per minute. This was followed by an equal volume of distilled H_2O. It was important that this strongly acidic Dowex 50 filtrate, which was clear and almost colorless, be immediately neutralized with 5N NH_4OH. This solution was then used for column chromatography on Dowex 1-X8 chloride (60 to 80 wet mesh) for separation of metabolites.

2. *Column chromatography of trichloroacetic acid extracts:* The extracts from the above procedure were then run through Dowex 1-X8 chloride (60 to 80 wet mesh) at about 3.5 ml. per minute. Resin columns of 1 by 14 cm. were used when the volume of red cells was less than 60 ml. Certain combinations of elution agents were found to separate various red cell metabolites which had been previously absorbed in the Dowex 1-X8 resin. The preceding was the elution specifications which were used for the columns of Dowex 1-chloride. The compounds absorbed on the small columns were eluted at a flow rate of 2.8 ml. per minute, collecting fractions of 20 ml. volume in the following pattern: 300 ml. 0.003N HCl, 300 ml. 0.01N HCl, 300 ml. 0.02N NH_4Cl, and 300 ml. 0.1N NH_4Cl. The curve in Fig. 2 was obtained.

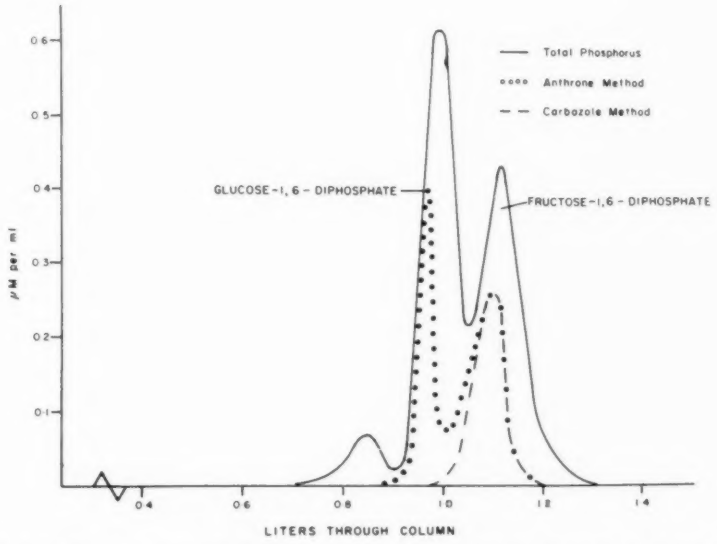

Fig. 3.—Diagram showing the glucose-1,6-diphosphate peak on Dowex 1-X8 formate column. The corresponding peaks for the cysteinecarbozole method and the anthrone method are also given.

The curve was plotted by employing phosphorus analysis. This was done in the following manner: 2.0 ml. sample from the 20 ml. fractions was added to 0.5 ml. 10N H_2SO_4, in a conical centrifuge tube, and was then heated in a 150° to 160° C. oven for 3 hours. Two drops of 30 per cent H_2O_2 was added and the tubes were returned to the oven for 1.5 hours; 4.6 ml. of 0.22 per cent ammonium molybdate was then added with 0.2 ml. Fiske-SubbaRow reagent (add 0.5 Gm. of purified 1-amino-2-naphthol-4-sulfonic acid, Eastman, with mechanical stirring to 200 ml. of freshly prepared 15 per cent anhydrous sodium bisulfite). The solution was then filtered and stored in a dark bottle. (It is prepared weekly.) The tubes are mixed thoroughly. This was then heated for 7 minutes in a boiling water bath with marbles covering the tubes. The O.D. is then read at 830 mμ in a Beckman spectrophotometer with a red-sensitive phototube.

The 0.1N NH_4Cl fraction was then run through a column of Dowex 1-X8 formate (100 to 325 wet mesh) 1 by 14 cm. column, which was eluted at 1.3 ml. per minute with a 2 L. of linear gradient 0 to 2.0N, pH 3.0 ammonium formate. Employing the phosphorus determination mentioned above, a cysteinecarbozole determination and an anthrone determination the curves in Fig. 3 were obtained.

Cysteinecarbozole method: To 2.0 ml. of sample from the 20 ml. fractions the following was added: 0.2 ml. freshly prepared 1.5 per cent cysteine-HCl, 4.0 ml. 95 per cent H_2SO_4 (with cooling in ice bath), and 0.2 ml. 0.1 per cent carbozole (Eastman) in absolute ethanol. This was heated for 15 minutes at 60° C. The O.D. was read, in 20 minutes at 560 mμ (1 cm. light path).

Anthrone method: To 2.0 ml. of sample obtained from the 20 ml. fractions, 4.0 ml. 0.2 per cent anthrone in 95 per cent H_2SO_4 was added and was heated for 10 minutes at 60° C., shaken well, and read at an O.D. 630 mμ when fructose was to be determined. When glucose was to be determined the samples were heated for 10 minutes at 100° C.

Preparation of Phosphoglucomutase.—The phosphoglucomutase was prepared by extraction of rabbit muscle.[13] The final extract was dialyzed in the cold for 48 hours. The enzyme solution was then passed through a column of Dowex-1-chloride and diluted 1:100 with cold distilled water just before use. Sigma glucose-1-phosphate was purified on a column of Dowex 1-X8 chloride.

Activation of Phosphoglucomutase by Red Cell Glucose-1,6-Diphosphate.—The reaction mixture contained 1 μM $MgSO_4$, 5 μM cysteine-HCl, 0.86 μM G-1-P, various dilutions of glucose-1,6-diphosphate, and 0.1 ml. of enzyme in a total volume of 0.6 ml., pH 7.5. After incubation for 10 minutes at 37.2° C., 0.4 ml. of 10N H_2SO_4 and 3.0 ml. of water were added, and the mixture was heated for 5 minutes at 100° C. and assayed for inorganic phosphorus. The enzymatic conversion of glucose-1-phosphate to glucose-6-phosphate was activated by glucose-1,6-diphosphate, and was measured by the decrease in hydrolyzable phosphorus.

RESULTS AND DISCUSSION

The galactose-1-phosphate uridyl transferase levels in the 14 galactosemic families using the four different assay methods are summarized in Tables I to IV.

TABLE I. GALACTOSE-1-PHOSPHATE URIDYL TRANSFERASE LEVELS BY UDPG CONSUMPTION TEST (METHOD A)

ACTIVITY (U./GM. HGB.)	HOMOZYGOTES	HETEROZYGOTES*	CONTROLS*
0-0.5	10	—	—
0.6-1.0	5	—	—
1.1-1.5	3	1	—
1.6-2.0	—	5	1
2.1-2.5	—	8	—
2.6-3.0	—	3	2
3.1-3.5	—	5	3
3.6-4.0	—	1	2
4.1-4.5	—	4	3
4.6-5.0	—	2	1
5.1-5.5	—	—	2
5.6-6.0	—	—	3
6.1-6.5	—	—	1
6.5-	—	—	7
Total	18	29	25
Mean	0.5	3.0	5.4
S. D.	±0.5	±1.0	±2.0
Range	0-1.5	1.1-5.5	1.8-9.9

*t = 5.4; d.f. = 52; p = 0.001.

TABLE II. GALACTOSE-1-PHOSPHATE URIDYL TRANSFERASE LEVELS BY MODIFIED UDPG CONSUMPTION TEST (METHOD B)

ACTIVITY (μM UDPG CONSUMED/ HR./GM. HGB.)	HOMOZYGOTES	HETEROZYGOTES*	CONTROLS*
0-1.0	9	—	—
1.1-2.0	—	—	—
2.1-3.0	1	1	—
3.1-4.0	—	3	—
4.1-5.0	2	3	—
5.1-6.0	—	5	—
6.1-7.0	—	1	—
7.1-8.0	1	1	—
8.1-9.0	—	1	—
9.1-10.0	—	1	1
10.1-11.0	—	2	—
11.1-12.0	—	—	1
12.1-13.0	—	—	2
13.1-14.0	—	1	1
14.1-15.0	—	—	1
15.1-16.0	—	1	2
16.1-	—	—	4
Total	13	20	12
Mean	1.6	6.8	15.2
S. D.	±2.2	±3.4	±3.5
Range	0-8.0	3.0-15.4	9.6-23.3

*t = 6.4; d.f. = 30; p = 0.001.

TABLE III. GALACTOSE-1-PHOSPHATE URIDYL TRANSFERASE LEVELS BY WARBURG MEASUREMENTS OF OXYGEN UPTAKE WITH SUPPLEMENTARY GLUCOSE-1,6-DIPHOSPHATE AND PHOSPHOGLUCOMUTASE

ACTIVITY (μl O_2/HR./0.3 ML. HEMOLYZATE)	HOMOZYGOTES	HETEROZYGOTES*	CONTROLS*
0-2.5	3	—	—
2.6-5.0	1	—	—
5.1-7.5	4	—	—
7.6-10.0	6	—	—
10.1-12.5	—	—	—
12.6-15.0	—	3	—
15.1-17.5	—	3	—
17.6-20.0	—	5	—
20.1-22.5	—	6	1
22.6-25.0	—	1	3
25.1-27.5	—	—	3
27.6-30.0	—	—	2
30.1-32.5	—	—	2
32.6-	—	—	3
Total	14	18	14
Mean	6.2	18.8	29.1
S. D.	±2.8	±3.1	±5.1
Range	1.1-9.0	12.9-23.1	21.7-40.7

*t = 6.4; d.f. = 30; p = 0.001.

The data show first that a small but significant amount of galactose-1-phosphate uridyl transferase activity can be detected among many of the homozygotes with all four methods. This is at variance with previously presented data.[2]

They further show that the mean value for a group of heterozygotes is significantly lower than for a group of normal control subjects using any of the

TABLE IV. GALACTOSE-1-PHOSPHATE URIDYL TRANSFERASE LEVELS BY WARBURG MEASUREMENTS OF OXYGEN UPTAKE WITHOUT SUPPLEMENTATION

ACTIVITY (μL O_2/HR./0.3 ML. HEMOLYZATE)	HOMOZYGOTES	HETEROZYGOTES*	CONTROLS*
0-2.5	2	—	—
2.6-5.0	3	—	—
5.1-7.5	3	—	—
7.6-10.0	6	—	—
10.1-12.5	—	—	—
12.6-15.0	—	4	—
15.1-17.5	—	3	—
17.6-20.0	—	7	—
20.1-22.5	—	3	1
22.6-25.0	—	—	2
25.1-27.5	—	1	4
27.6-30.0	—	—	2
30.1-32.5	—	—	1
32.6-	—	—	4
Total	14	18	14
Mean	6.0	18.2	29.2
S. D.	±2.9	±3.5	±5.3
Range	0.4-9.4	13.9-26.4	21.7-40.0

*t = 6.4; d.f. = 30; p = 0.001.

four methods. Although the overlap between the heterozygotes and normal control subjects is greater by the UDPG consumption tests (methods A and B) as compared with the Warburg measurements of oxygen uptake (methods C and D), the difference between the means as measured by the "t" test is not significant. Furthermore, some degree of overlap exists even by the latter method.

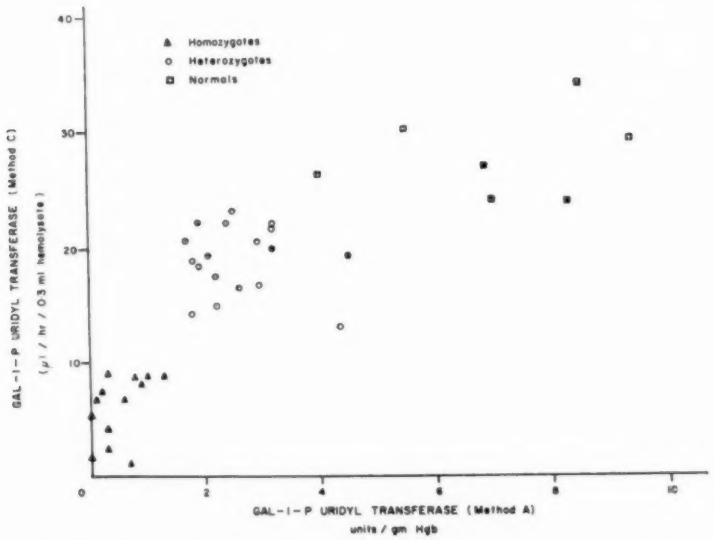

Fig. 4.—Comparison of galactose-1-phosphate uridyl transferase levels using the UDPG consumption test (method A) and the oxygen uptake method (method C) in normal control subjects, heterozygotes, and homozygotes. The correlation coefficient (r) = +0.82 (76 degrees of freedom).

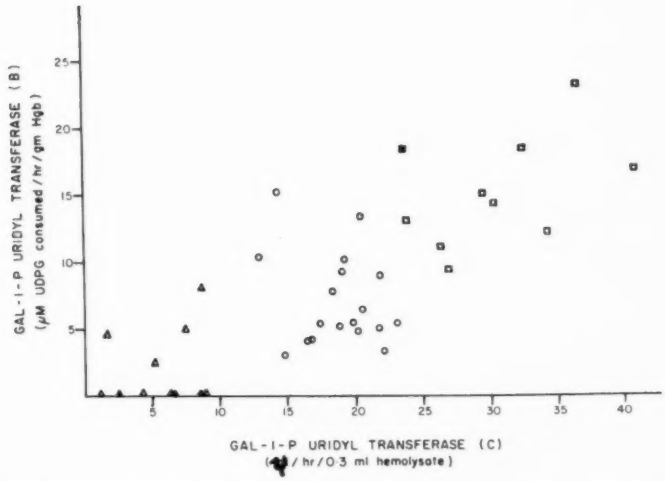

Fig. 5.—Comparison of galactose-1-phosphate uridyl transferase levels using the modified UDPG consumption (method B) and the oxygen uptake method (method C) in normal control subjects, heterozygotes and homozygotes. The correlation coefficient (r) = +0.79 (80 degrees of freedom).

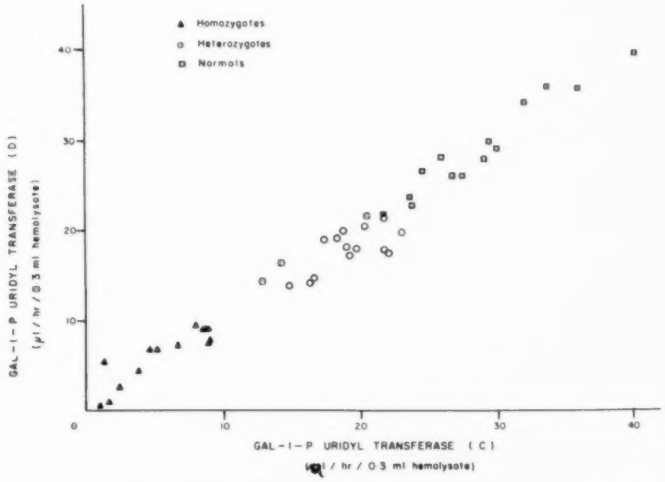

Fig. 6.—Comparison of galactose-1-phosphate uridyl transferase levels using the oxygen uptake method with added phosphoglucomutase and glucose-1,6-diphosphate (method C) and without supplementation (method D) in normal control subjects, heterozygotes, and homozygotes. The correlation coefficient (r) = +0.98 (88 degrees of freedom).

We have had the opportunity of comparing the galactose-1-phosphate uridyl transferase system using the oxygen consumption test with added phosphoglucomutase and glucose-1,6-diphosphate (method C) with the other three methods in the same individuals. It was found that a fair correlation exists between this method and the UDPG consumption test (method A), the correlation coefficient being (r) = +0.82 (76 degrees of freedom) as shown in Fig. 4. A similar

correlation exists between this method and the modified UDPG consumption test (method B), the correlation coefficient being (4) = +0.79 (80 degrees of freedom) as shown in Fig. 5. Finally, an excellent correlation exists between this method and the same oxygen consumption method without supplementary compounds, the correlation coefficient being (r) = +0.98 (88 degrees of freedom) as shown in Fig. 6. This would indicate that the oxygen consumption method with supplementary phosphoglucomutase and glucose-1,6-diphosphate described by Kirkman and Bynum[10] is not the only method available for the detection of heterozygotes in galactosemia.

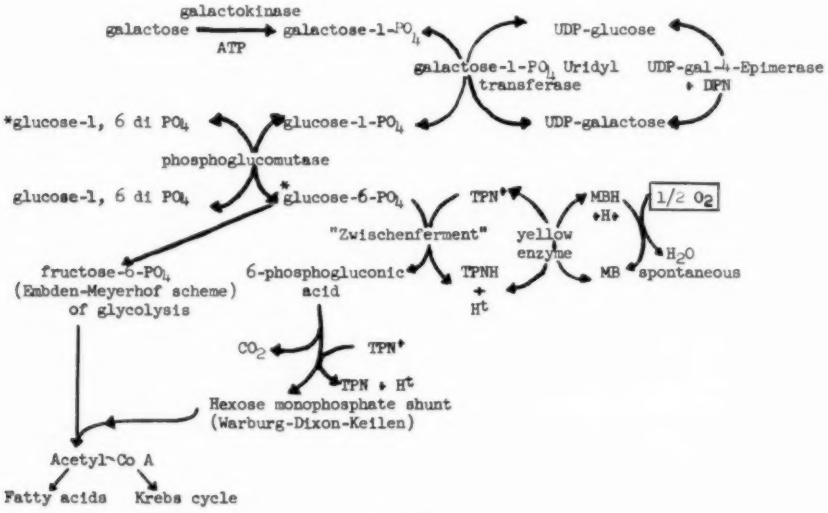

Fig. 7

While undertaking kinetic studies, a consistent lag period was noted in three of the assay procedures (methods B, C, and D) used to estimate the various levels of galactose-1-phosphate uridyl transferase. This finding has also been noted by Schwarz.[14] This lag should be considered in the light of the pathway responsible for the utilization of galactose, which is as follows:

It would appear that the lag is not caused by a deficiency of either phosphoglucomutase or glucose-1,6-diphosphate. In the first place, the oxygen uptake by the Warburg procedure was the same whether the supplementary compounds were added or not. Also, it was found that a tenfold increase of glucose-1,6-diphosphate and/or phosphoglucomutase did not significantly increase oxygen uptake.[15] Finally, with the use of column chromatography, it was found that the content in normal adult red cells of glucose-1,6-diphosphate per 0.3 ml hemolyzate was 0.10 μM mean concentration, whereas the amount that was added in the reaction was 0.0014 μM. This leads one to suspect that the addition of glucose-1,6-diphosphate, and possibly of phosphoglucomutase, was not essential and that these are not the limiting factors in this system.[16]

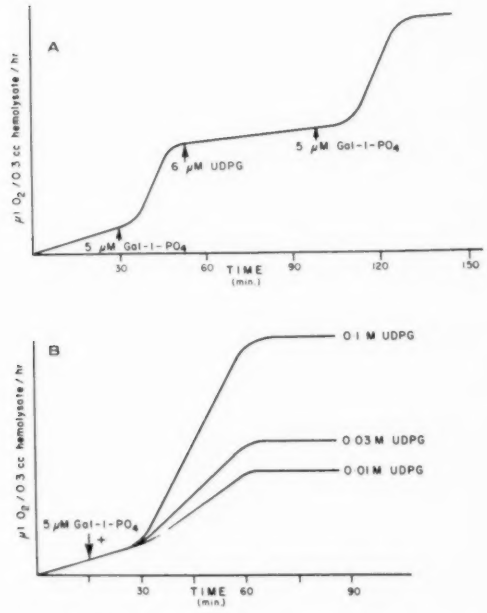

Fig. 8.—The effect of added UDPG upon oxygen uptake. *A*, 6 μM of added UDPG has no influence upon uptake whereas 5 μM of Gal-1-P increases uptake. *B*, A tenfold increase of added UDPG only increases uptake twofold.

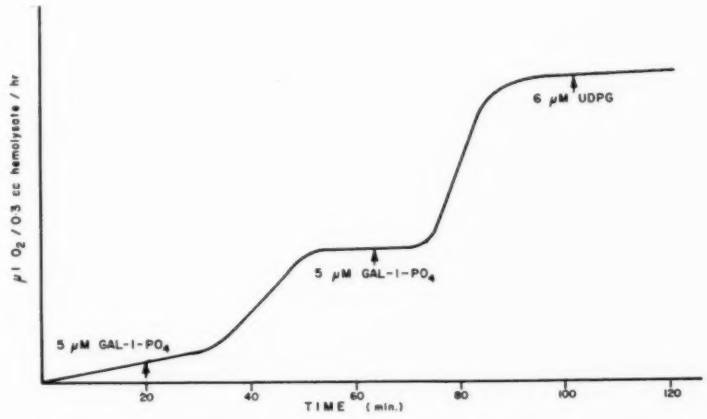

Fig. 9.—Diagram showing that the addition of 5 μM of Gal-1-P results in increased oxygen uptake in two instances, whereas the addition of 6 μM of UDPG has no effect.

Similarly, the lag does not appear to be related to the concentration of UDPG (Figs. 8 and 9). If one increases the concentration of UDPG tenfold and alters the rate of oxygen uptake, there is no decrease in the lag period (Fig. *B*). Also, supplementary additions of galactose-1-phosphate were taken through the "trapping system" without the necessity of adding more UDPG

which indicated that there is enough epimerase present to regenerate UDPG which had been consumed within the same flask in the previous reaction (Fig. 9).

Finally, the control flasks which contained glucose-1-phosphate did not show a similar lag which indicated that a consistent pathway is still present and not blocked by galactose-1-phosphate, which was absent.

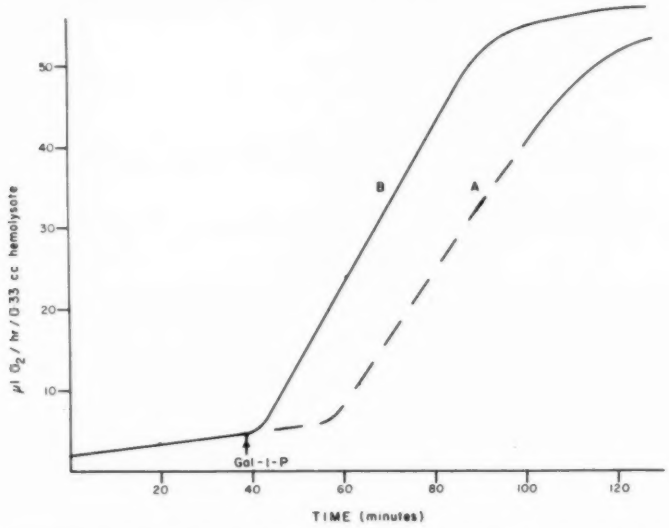

Fig. 10.—Experiments showing the effect of the addition of glucose-6-phosphate dehydrogenase upon the galactose-1-phosphate uridyl transferase system. *A*, The typical lag before the initiation of oxygen uptake is noted when no supplementary glucose-6-phosphate dehydrogenase was added. *B*, The lag is no longer present when supplementary glucose-6-phosphate dehydrogenase was added.

The lag period encountered in the reconstructed systems was corrected by supplementation with glucose-6-phosphate dehydrogenase (Zwischenferment). This phenomena was observed whether this enzyme was preincubated in the Warburg flasks before the addition of galactose-1-phosphate, or when it was tipped in simultaneously with the substrate (Fig. 10). Since the over-all consumption of oxygen was not affected by the addition of Zwischenferment, the lag must represent either an actual deficiency of glucose-6-phosphate dehydrogenase, or that there is competitive inhibition with galactose-1-phosphate in the trapping system.[15, 16]

SUMMARY

The present article describes the measurement of galactose-1-phosphate uridyl transferase activity by four different methods of assay.

In fourteen galactosemic families, it was shown that the mean value for a group of heterozygotes is significantly lower than for a group of normal control subjects when any of the four methods was used. Although the overlap between the groups is greater by the uridine diphosphoglucuronic acid (UDPG) consumption tests as compared with the Warburg measurements of oxygen uptake, the difference between the means as measured by the "t" test is not significant.

In the course of these studies, a consistent lag period was noted after the addition of galactose-1-phosphate. It was shown that the concentrations of phosphoglucomutase, glucose-1,6-diphosphate, uridine diphosphoglucose, galactose-1-phosphate, and epimerase were not the limiting factors in this system. On the other hand, the lag could be corrected by supplementation with glucose-6-phosphate dehydrogenase. This would indicate that there is either an actual deficiency of glucose-6-phosphate dehydrogenase, or there is competitive inhibition with galactose-1-phosphate in this system.

We wish to express our deep appreciation to the members of the families for being helpful and cooperative in every way and to the following physicians for referring patients to us for this study: Drs. Audrey Brown, Eugene Diamond, Samuel Foman, Minnie Frank, Alvah L. Newcomb, Normal Pokorny, and Edward Stabins. We would also like to acknowledge helpful advice given by Drs. V. Schwarz and H. Harris and the assistance of Drs. Shirley G. Driscoll, and Kenneth Hugh-Jones, and June Cosbey in collecting the samples.

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ELECTROPHORETIC PARTITION OF CEREBROSPINAL FLUID AND SERUM PROTEINS IN MULTIPLE SCLEROSIS

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The distribution of the proteins of the cerebrospinal fluid and serum of 23 patients with multiple sclerosis and 6 control patients was studied by filter paper electrophoresis. The partition of the serum proteins was normal in all patients. The partition of cerebrospinal fluid proteins of 6 patients with multiple sclerosis and an abnormal Lange gold curve reaction showed a markedly significant increase of the gamma globulin and decrease of the albumin concentration, and differed strikingly from simultaneously studied serum proteins. The partition of cerebrospinal fluid proteins was normal in all other patients studied and was similar to the serum proteins. The duration of illness in patients with an increased cerebrospinal fluid gamma globulin concentration was significantly decreased. The increase in gamma globulin concentration in these patients may be due to infiltration of the central nervous system by plasma cells early in the course of multiple sclerosis.

A MORE precise characterization of the distribution of the cerebrospinal fluid protein fractions is provided by the recent development of methods for preliminary concentration of small quantities of protein-poor fluids so that reproducible paper zone electrophoresis can be accomplished as readily as for serum proteins.[1, 2] In this article, the simultaneous electrophoretic partitions of the cerebrospinal fluid and serum proteins of patients with multiple sclerosis will be presented and correlated with their clinical status. This study forms part of an inquiry into the protein composition of the cerebrospinal fluid in disorders of the central nervous system and the state of permeability of the blood–cerebrospinal fluid barrier.[3]

METHODS

Twenty-three patients were studied. All presented histories, physical signs, and laboratory findings compatible with the diagnosis of multiple sclerosis. None had serologic evidence of syphilis. Nineteen patients were long-standing inhabitants of a hospital for chronic disease, and four were observed on the neurologic service of a large general hospital. Each patient was graded clinically according to the disability scale of Kurtzke and Berlin.[4] This and additional clinical data are presented in Table I.

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TABLE I. CLINICAL DATA IN 23 PATIENTS WITH MULTIPLE SCLEROSIS

CASE NO.	SEX	AGE (YR.)	AGE AT ONSET (YR.)	DURATION OF ILLNESS (YR.)	DURATION PRESENT SYMPTOMS (YR.)	DISABILITY SCALE (KURTZKE-BERLIN)	COLLOIDAL GOLD CURVE
1	M	39	35	4	1/12	4	Abnormal
2	M	40	31	9	9	7	Abnormal
3	M	58	44	14	4	7	Abnormal
4	F	51	42	9	9	6	Abnormal
5	F	48	47	3/4	3/4	7	Abnormal
6	F	49	49	1/12	1/12	8	Abnormal
7	F	42	33	9	9	9	Normal
8	M	44	29	15	12	7	Normal
9	F	41	32	9	4	7	Normal
10	M	44	39	5	2	7	Normal
11	M	27	19	8	8	7	Normal
12	M	46	43	3	3	7	Normal
13	F	42	36	6	6	7	Normal
14	M	55	36	19	19	7	Normal
15	M	52	44	8	2	9	Normal
16	M	52	45	7	7	9	Normal
17	M	41	30	11	11	9	Normal
18	M	45	23	22	4	8	Normal
19	F	52	24	28	1/12	7	Normal
20	M	41	35	6	5	7	Normal
21	M	46	43	3	3	7	Normal
22	F	44	10	34	3	7	Normal
23	F	35	25	10	5	7	Normal
Average for abnormal gold curve	F3 M3	48	41	6	4	7	6
Average for normal gold curve	F6 M11	44	33	12	6	8	17

The control patients consist of five men and one woman, 48 to 86 years old, average age 62 years, from whom cerebrospinal fluid and serum were obtained just prior to the induction of spinal anesthesia for elective surgery (for hernia, hypertension, femoral fracture, and arterial insufficiency). None had evidence of neurologic disease.

Preliminary concentration and electrophoresis of the cerebrospinal proteins were performed as previously described.[3] Briefly, about 10 ml. of cerebrospinal fluid is dialyzed through cellophane against 20 per cent polyvinylpyrrolidone for 16 to 24 hours to a volume of 0.2 ml. Paper electrophoresis is then performed as for serum proteins. Greater experience with this method has demonstrated that: (1) all protein fractions identified in serum can be detected in the cerebrospinal fluid; (2) the alpha$_1$ globulin fraction is sometimes too small to be clearly identified; and (3) a clear-cut separation of the alpha$_2$ and beta globulin fractions is occasionally difficult. Furthermore, certain protein fractions are observed in the cerebrospinal fluid which are not ordinarily observed in the serum. These are: (1) a prealbumin fraction, with a mobility greater than that of albumin, observed in the majority of cases,[5] (2) a fraction intermediate in mobility between the beta and gamma globulins designated here as the beta$_2$ globulin fraction,[6] and (3) a starting line artifact due to denaturation of protein during the concentration procedure.[7] The latter seldom exceeds 1 per cent of the total protein content so that preliminary dialysis against saline to prevent increased salt concentration and lessen the degree of denaturation is unnecessary. A satisfactory separation of protein fractions can be obtained in 92 per cent of specimens. When

TABLE II. MEAN AND STANDARD DEVIATION OF ELECTROPHORETIC PROTEIN FRACTIONS IN CEREBROSPINAL FLUID AND SERUM IN PATIENTS WITH MULTIPLE SCLEROSIS AND CONTROL PATIENTS

SOURCE	DIAGNOSTIC GROUP	NO. OF PATIENTS	TOTAL PROTEIN (GM./100 ML.)	PROTEIN PARTITION (%)					
				PRE-ALBUMIN	ALBUMIN	α_1-GLOBULIN	α_2-GLOBULIN	β-GLOBULIN	γ-GLOBULIN
Cerebrospinal fluid	Multiple sclerosis with abnormal gold curve	6	0.039 ± 0.013	1.0	36 ± 6.8	4 ± 1.4	6 ± 1.5	15 ± 2.4	38 ± 8.8
	Multiple sclerosis with normal gold curve	17	0.043 ± 0.028	1.6	50 ± 9.2	5 ± 1.8	10 ± 3.8	15 ± 3.0	19 ± 4.6
	Controls	6	0.031 ± 0.010	1.0	45 ± 5.3	6 ± 3.1	9 ± 2.1	21 ± 4.3	18 ± 4.0
Serum	Multiple sclerosis with abnormal gold curve	6	7.5 ± 0.24		43 ± 4.4	6 ± 2.1	12 ± 2.1	15 ± 2.2	24 ± 7.2
	Multiple sclerosis with normal gold curve	17	6.7 ± 0.66		48 ± 5.4	6 ± 2.1	12 ± 2.8	14 ± 2.4	20 ± 5.0
	Controls	6	6.6 ± 0.52		42 ± 5.8	5 ± 1.6	13 ± 3.1	18 ± 2.3	22 ± 3.3

the amount of cerebrospinal fluid is adequate, failure can be attributed to extremely low protein concentration, insufficient concentration, or concentration to dryness with subsequent inability to locate protein in the recesses of the cellophane bag.

The colloidal gold curve reaction was performed according to the method of Lange.[8] The spinal fluid total protein concentration was determined by the sulfosalicylic acid method.[9]

For purposes of statistical analysis, the patients were divided into three groups: patients with multiple sclerosis and an abnormal colloidal gold curve reaction, patients with multiple sclerosis and a normal colloidal gold curve reaction, and control subjects without neurologic disease. The mean and standard deviation for total protein and electrophoretic fractions of cerebrospinal fluid and serum of these groups were determined by standard methods (Table II). Differences between groups were analyzed by the "t" test for non-paired samples (Table III).

RESULTS

Serum Proteins.—(Tables II and III.) A significant increase in concentration of total serum protein was found in the patients with multiple sclerosis who had an abnormal gold curve reaction. The electrophoretic partition of serum proteins of this group, however, showed no significant difference when compared to the groups with negative gold curve reactions. The patients with multiple sclerosis who had a normal gold curve reaction showed a significant decrease of serum beta globulins and increase of serum albumin concentrations when compared to the control patients.

Cerebrospinal Fluid Proteins.—(Tables II and III.) No significant differences in total protein concentration was found for the three groups. The spinal fluid protein concentration was between 20 and 50 mg. per 100 ml. in all but four patients. The majority of the patients with multiple sclerosis had a positive Pandy reaction regardless of the total protein concentration or the type of gold curve reaction. The cerebrospinal fluid cell count and dynamics were normal in all patients.

A highly significant increase of the gamma globulin concentrations were found in the patients with multiple sclerosis who had an abnormal gold curve reaction. This was accompanied by a highly significant decrease in the concentrations of albumin. The protein partition of patients with multiple sclerosis who had a normal gold curve reaction showed a significant decrease of the beta globulin concentrations when compared to those of the control patients.

TABLE III. TESTS OF SIGNIFICANCE: THE "t" TEST

COMPARISON NO.	TOTAL PROTEIN	ALBUMIN	α_1-GLOBULINS	α_2-GLOBULINS	β-GLOBULINS	γ-GLOBULINS
I		0.25	1.36	3.55*↓	0.82	1.32
II	0.12	3.68*↓	0.95	0.85	0.20	4.84*↑
III	1.75	1.58	0.60	0.55	3.18†↓	0.50
IV	4.00*↑	1.31	0.29	0.00	0.02	0.97
V	1.07	2.20‡↑	0.09	0.77	3.35†↓	1.10

Comparisons: I = Cerebrospinal fluid versus serum; all groups. II = Cerebrospinal fluid: abnormal versus normal gold curve. III = Cerebrospinal fluid, normal gold curve; multiple sclerosis versus controls. IV = Serum: abnormal versus normal gold curve. V = Serum, normal gold curve; multiple sclerosis versus controls.

Upright arrow indicates significant increase, down arrow indicates significant decrease.

*Significant at 0.1 per cent level.

†Significant at 1 per cent level.

‡Significant at 5 per cent level.

Comparison of Serum and Cerebrospinal Proteins.—(Tables II and III.) A significant decrease in the cerebrospinal fluid alpha$_2$ globulin concentration as compared to that of the serum was observed in all groups. A decrease of the concentration of beta globulins of both cerebrospinal fluid and serum in the patients with multiple sclerosis with or without an abnormal gold curve reaction was evident when compared to that in the control patients.

Clinical Correlation.—(Table I.) Both groups with multiple sclerosis exhibited severe disability when graded by the Kurtzke-Berlin scale. The single exception (Case 1) was a patient with an abnormal gold curve reaction. The duration of illness was less than one year in only two patients, both of whom had abnormal gold curve reactions. Only one patient (Case 6), with neuromyelitis optica (Devic's disease), could be classified as having acute multiple sclerosis. The average duration of disease was 6 years in the patients with an abnormal gold curve reaction and 12 years in the patients with a normal gold curve. In general, the patients with an elevated cerebrospinal fluid gamma globulin appeared to present a more active and earlier stage of disease than did the others. Additional clinical data are presented in Table I.

To evaluate duration of disease as a factor which influences cerebrospinal fluid gamma globulin concentration, the patients with multiple sclerosis and a normal gold curve reaction were subdivided into a group of five patients with illness of less than 6 years' duration and 12 patients with illness of 7 to 34 years' duration. A comparison of cerebrospinal fluid protein fraction concentrations revealed no significant differences.

DISCUSSION

The serum proteins in multiple sclerosis have been studied by many investigators with varying and sometimes conflicting results.[10-20] These variations are the result, most probably, of differences in methods. If only studies in which the techniques of zone electrophoresis are used are reviewed, most authors report a significant decrease in the serum albumin and an increase in the alpha$_2$ globulin concentrations as compared to those in normal subjects. Had we also used as controls normal young adults,[21] rather than hospitalized patients, the same results would have been obtained. Such changes have been found in a large variety of diseases.[22] They have been described as nonspecific in character comprising a generalized response to illness or to metabolic deterioration.[17, 23] It is not clear why, in this study, the patients with multiple sclerosis and an abnormal gold curve reaction had an increase in total serum protein concentration. The absence of any significant difference between the serum protein partitions of the two subgroups of multiple sclerosis confirms the findings of Press.[18]

The cerebrospinal fluid proteins in multiple sclerosis have been studied, most recently, by immunochemical,[24-26] flocculation,[11] chromatographic,[27] turbidimetric,[28, 29] and ultracentrifugal[18] methods as well as by free[16] and filter paper zone electrophoresis.[17, 18, 30-35] There is general agreement that an increase in the concentration of gamma globulins in cerebrospinal fluid occurs often in

multiple sclerosis. The reported incidence, however, varies from 24 to 80 per cent.[11, 36] There is also agreement that a normal concentration of gamma globulins in cerebrospinal fluid occurs not infrequently in typical instances of the disease.

A review of the results obtained for cerebrospinal fluid proteins by zone electrophoresis does not eliminate variations from differences in methods as readily as for serum proteins. The reported protein distribution for normal cerebrospinal fluid[2, 37] varies greatly, probably as a result of the differences in procedures used to concentrate the proteins. Kabat and co-workers[16] studied the cerebrospinal fluid of five patients with multiple sclerosis. An elevation of the gamma globulins and a decrease of the albumin concentration was observed in the one patient in their series with an abnormal gold curve reaction. Theirs was the only study in which the technique of free electrophoresis was used. In the following studies filter paper electrophoresis was used. Roboz and associates[17] studied ten patients, all of whom had multiple sclerosis of long duration. Their gold curve reactions were not recorded. A significant decrease in the albumin and an increase in the beta and gamma globulin fractions were observed as compared to those in normal subjects. When simultaneously performed serum and cerebrospinal fluid electrophoretic diagrams were examined by inspection a pronounced difference was observed in four of seven patients. Press[18] studied thirty-five patients. The cerebrospinal fluid protein changes were correlated with the Lange colloidal gold reaction, but no clinical data were recorded. The concentration of gamma globulin in 17 patients with an abnormal gold curve was 26.2 ± 7.7 per cent and in 18 patients with a normal gold curve 17.7 ± 2.4 per cent of the total protein. The albumin concentration was slightly decreased in patients with an abnormal gold curve. The other protein fractions were not recorded in detail. Needham and collaborators[30] found the relative gamma globulin concentration to range from 31 to 34 per cent in four patients with an abnormal gold curve reaction and from 11 to 22 per cent in 17 patients with a normal gold curve reaction. They did not record the other protein fractions. In contrast to these investigators, Cummings[31] found that the Lange reaction did not exactly parallel the amount of gamma globulin present in the cerebrospinal fluid. The results of the four electrophoretic diagrams he obtained were evaluated by inspection. An increase in cerebrospinal gamma globulin in the absence of a comparable change in the serum has also been noted by others,[32-35] but without reference to the Lange colloidal gold reaction.

In this study, the principal protein abnormality of the cerebrospinal fluid in multiple sclerosis consisted of an increase in the gamma globulin and a decrease in the albumin concentration in patients with an abnormal gold curve reaction. In contrast, the distribution of the serum proteins in these patients was normal. The protein partition of the cerebrospinal fluid and serum in patients with a normal gold curve reaction was normal, and resembled that of the control subjects. These findings agree in general with the results of the aforementioned investigators.

The increased cerebrospinal fluid gamma globulin content of some patients with multiple sclerosis has been ascribed to the formation of gamma globulin within the tissues of the central nervous system.[16] In 1942, Bing and Neel[39] suggested that the plasma cell within the central nervous system was the source of the gamma globulin. This is in accord with the modern concept that the plasma cell is the site of gamma globulin production.[40] This cell appears in moderate numbers in the cellular infiltrations found in some patients with multiple sclerosis.[41, 42] Lichtenstein[42] states that such accumulations of cells are more likely to occur during the acute stages of the disease. This would be in agreement with our observation that the increase in gamma globulin concentration of the cerebrospinal fluid occurs in the earlier, more active stages of multiple sclerosis. On the other hand, Field[43] found that the elevation of gamma globulin in the cerebrospinal fluid was correlated with an increase in the lymphocyte content. Frequent attempts to correlate the abnormal cerebrospinal fluid findings in multiple sclerosis with the clinical course and pathologic findings have produced no consistent results.[2, 36]

SUMMARY

The distribution of the proteins of the cerebrospinal fluid and serum of patients with multiple sclerosis and control patients without neurologic disease was studied by filter paper electrophoresis. Partition of the cerebrospinal fluid proteins demonstrated an increase in the gamma globulins and a decrease in the albumin concentration of those patients with multiple sclerosis who had an abnormal Lange gold curve reaction. In these patients, the distribution of the cerebrospinal fluid proteins differed strikingly from simultaneously studied serum proteins. The distribution of the cerebrospinal fluid proteins in the patients with multiple sclerosis who had a normal gold curve reaction did not differ significantly from those of the control patients and resembled the partition of simultaneously obtained serum proteins. The partition of serum proteins in all patients with multiple sclerosis was essentially unchanged from that of the control patients. The duration of illness in patients with an increased concentration of cerebrospinal fluid gamma globulin was significantly less than for the other patients.

The increase of gamma globulins in cerebrospinal fluid may be due to its formation by plasma cells which infiltrate the tissues of the central nervous system during the earlier more active stages of multiple sclerosis.

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INFRARED ANALYSIS OF SERUM PROTEIN FROM ONE HUNDRED AND FIVE HEALTHY ADULTS

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Representative infrared spectra of normal blood serum and of serum protein fractions from 105 healthy adults are presented to demonstrate that infrared analysis adds a new qualitative dimension to the investigation of the serum proteins. Two drops of blood serum or a serum protein fraction were analyzed in a double beam infrared spectrometer.[2] A range of qualitative differences in the various normal albumin fractions and in each of the three globulin fractions was readily apparent. Additional qualitative differences were observed in serum fractions from patients with neoplasms. In contrast, the range of variation in the spectra of normal blood serum was small, with most differences limited to the 7.0 to 7.5 μ region.

THE initial experiments dealing with the technique of obtaining infrared spectra of blood sera[1] demonstrated that infrared spectroscopy offers a rapid method for the analysis of blood serum. Infrared spectra of blood sera from patients with established diagnoses were presented to show the dramatic sensitivity to differences in serum protein. However, before spectra of series of sera or their protein fractions from a significant number of patients with a single disease are examined, it is necessary that a series of normal blood sera samples and serum protein fractions from these be analyzed to ascertain the range of the normal pattern. This article is the report of the infrared analysis of such samples from 105 healthy adults.

MATERIALS AND METHODS

Infrared Method.—The infrared spectrometer used for this study was the improved optical null instrument described by Herscher and coauthors.[2] The details of the method have been described previously, so only a brief description is given here. Two drops of the sample are spread out between two BaF_2 crystal windows and placed in the sample beam of the spectrometer. Either distilled water or pooled serum in a similar cell is placed in the reference beam of the spectrometer. When scanned with water as a reference the strong absorption bands of the proteins are observed. When scanned with pooled serum as a reference the common absorption bands are cancelled and only differences between the sample and reference are recorded. The total time required to obtain the infrared spectrum is approximately 15 minutes. During 12 of these minutes the spectrometer is automatically

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recording the spectrum, and the operator is free to prepare the succeeding sample. The spectra presented are photographs of original tracings produced automatically by the infrared spectrometer. Wavelength in microns is indicated on the abscissa, and absorbency is plotted on the ordinate.

Serum Samples.—Serum from venous blood was obtained from 105 healthy adults, ages 17 to 45 years, with a mean age of 24 years. Prior to selection as a normal subject, a complete history was obtained and a thorough physical examination was performed. Each subject had the following clinical examinations: chest x-ray, timed vital capacity, orthorater, audiogram, Kahn test, hemoglobin, white blood cell count, urinalysis, and other indicated clinical blood tests.

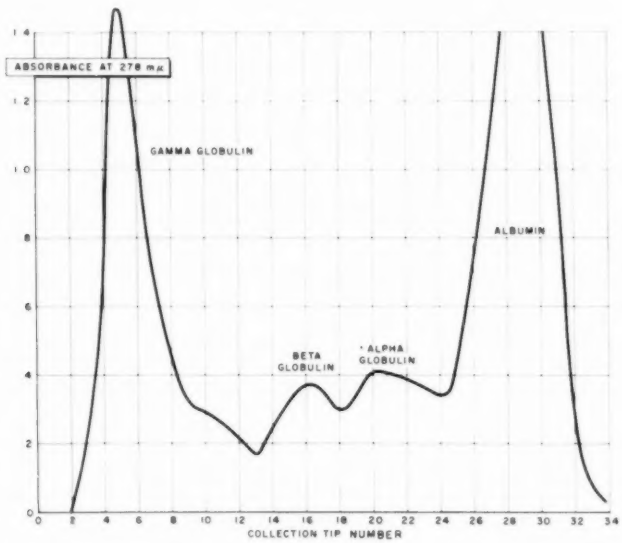


Fig. 1.—Absorption at 278 mμ of electrophoretic collection tip fractions from normal blood serum.

Group A: Eight sets of ten sera each.

1. Each serum sample was scanned differentially with the serum in the sample beam and with sera pooled from its group of ten samples in the reference beam.

2. Each of the eighty serum samples was scanned with distilled water in the reference beam.

3. Electrophoretic separation of the serum proteins for infrared analysis was performed upon twelve of the samples.

4. Seventeen of the samples were concentrated five times by weight by evaporation under vacuum. After this they were scanned differentially against a concentrated pooled control serum sample and against distilled water.

Group B: Twenty-five sera were scanned with distilled water in the reference beam.

Separation of Serum Protein Fractions by Continuous Flow Curtain Electrophoresis.—A Spinco model CP continuous flow paper electrophoresis cell with automatic fraction collector was used. A buffer solution, pH 8.6, was prepared by dissolving 61.8 Gm. of boric acid and 9.6 Gm. of sodium hydroxide pellets in 20 L. of distilled water. A 10 mm. diameter sample tube was filled with approximately 5 ml. of human blood serum. The height of the buffer in the reservoir was set at 2.5 scale units. Water at a temperature of approximately 10° C. was circulated through the cooling plate.

After allowing 3 to 4 hours for the instrument to come to equilibrium with the power supply set at 860 volts, the sample flow was started at a rate of 0.4 ml. per hour on a tab 2 inches from the negative side of the curtain. Thirty minutes after the fastest moving component reached the bottom of the curtain, collection of serum protein fractions from the thirty-two tips was begun. These were collected for 8 to 12 hours.

Alternate fractions of the thirty-two which were collected were scanned on a Cary recording spectrophotometer model A in the 250 to 310 mμ region. A typical plot of the absorption at 278 mμ of the normal fractions is presented in Fig. 1. The albumin and gamma globulin fractions were easily identified, whereas the alpha and beta globulin fractions, having less absorption at 278 mμ, were less easily identified. In most instances it was impossible to separate sharply $alpha_1$ and $alpha_2$ globulin fractions. Separate samples of albumin and gamma globulin were fractionated under identical conditions. The plot of the absorption at 278 mμ is shown in Fig. 2. This use of the ultraviolet absorption was superior to curtain staining for identification of the various serum fractions.

The fractions were combined into four groups: albumin, alpha globulin, beta globulin, and gamma globulin. These were concentrated to 0.75 ml. in a rotating evaporator flask connected to a vacuum pump. The samples were then dialyzed against 0.9 per cent saline to remove the borate buffer which has strong broad absorption bands at 7, 7.5, 8.65, and 10 μ.

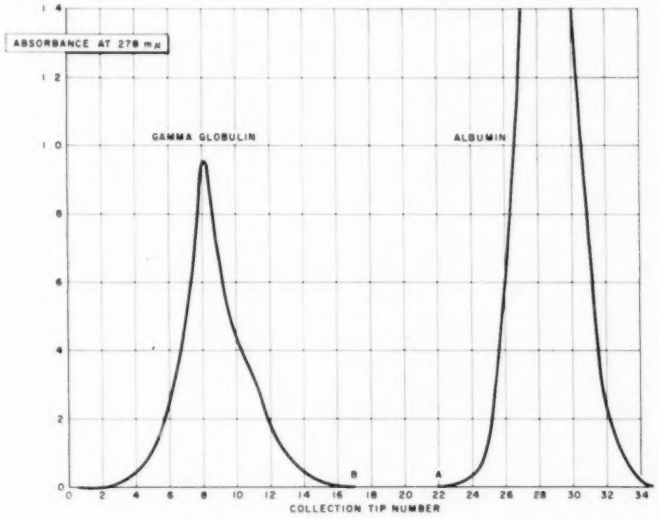


Fig. 2.—Absorption at 273 mμ of electrophoretic collection tip fractions. *A*, Pure albumin. *B*, Pure gamma globulin.

RESULTS

Normal Serum Scanned With Water in the Reference Beam.—Of the 105 infrared spectra obtained, 104 showed essentially the same pattern between 7.5 and 10.0 μ, with minor variations observed below 7.5 μ. Fig. 3 is the infrared spectrum of a representative serum sample. The absorption bands are due to serum protein which masks both serum carbohydrate and lipid constituents when these are present in normal concentrations. The molecular structures which give rise to these absorption bands remain to be determined.

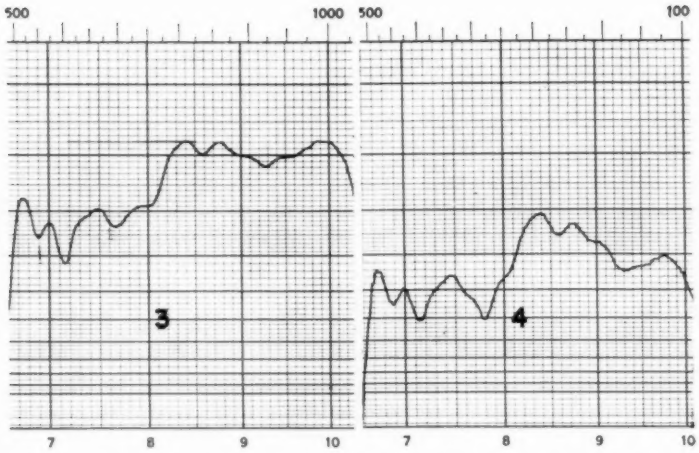


Fig. 3.—Infrared spectrum obtained most frequently from the blood serum of healthy adults. Serum scanned with distilled water in the reference beam.

Fig. 4.—Infrared spectrum of blood serum from a 22-year-old woman showing absorption at 7.8 μ. Serum scanned with distilled water in the reference beam.

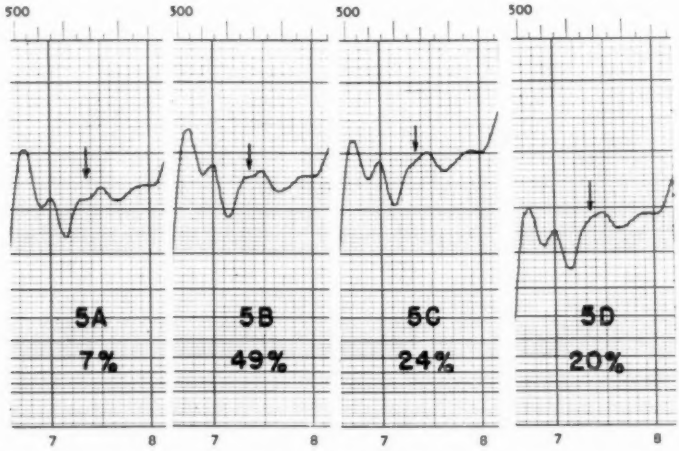


Fig. 5.—Typical infrared spectra showing the variations in the normal blood serum pattern at 7.35 μ.

The one serum which showed a marked difference from the other spectral patterns was obtained from a 22-year-old woman. This spectrum (Fig. 4) has a prominent absorption band at 7.8 μ.

The variations observed below 7.5 μ fall into four different groups, principally characterized by their absorption between 7.3 and 7.4 μ. Fig. 5 shows this area in four representative spectra. Pattern *A* was present in 7 per cent of the sera, pattern *B* in 49 per cent, pattern *C* in 24 per cent, and pattern *D* in 20 per cent. Serial spectra of serum obtained from the same individual at weekly intervals revealed that the pattern in this region is not constant. Pattern *B*, present one week, may be replaced, for example, by pattern *C* the following week. The reason for these variations has not been studied.

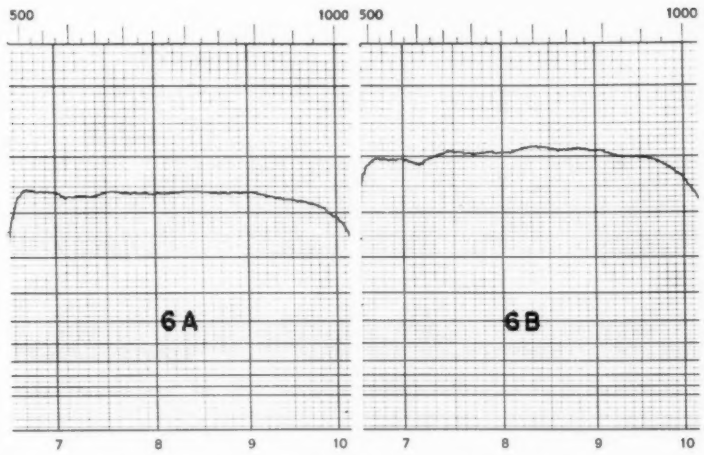


Fig. 6.—Differential scans between two pairs of normal blood sera. Serum scanned with pooled sera sample in the reference beam.

Normal Serum Scanned With Pooled Normal Serum in the Reference Beam.—Eighty differential scans were run with a normal serum in the sample cell and with serum pooled from ten individuals in the reference cell. The normal serum sample constituted one tenth of the pooled sample. Seventy-three of the differential scans showed no significant new absorption bands as have been observed in pathologic sera spectra.[1] Two representative differential scans from this normal group are presented in Fig. 6. The smaller deflections visible are due to instrument background noise. In Fig. 6A the downward deflection at 7.1 to 7.2 μ indicates that more of a particular protein complex was absorbing energy in the sample beam than was being absorbed in the reference beam. This is due to a slight mismatch of sample and reference absorption, arising either from small concentration differences, or from a difference between the cell thickness of the sample serum and that of the reference serum. This same absorption also appears in Fig. 6B, and since its ratio to the other serum absorption bands is the same as that found when scanning pooled serum versus distilled water, it is interpreted as arising from cell mismatch and not from an abnormality in the serum.

The seven sera in which pattern *A* was observed showed a band between 3 and 7.4 μ when scanned differentially confirming this observation in the ans versus distilled water. The band at 7.8 μ was also confirmed in the serum ectrum of the 22-year-old woman mentioned previously.

Infrared Analysis of Serum Protein Fractions Obtained by Curtain Electro-oresis.—

Albumin: Fig. 7 is the infrared spectrum of a pooled sample of human bumin obtained from the Michigan Department of Health. Fig. 8 is a repre-tative spectrum of albumin from the serum of an individual in this normal ies. Although the sample in Fig. 8 is less concentrated than the one in Fig. 7,

its infrared spectrum is identical. Definite broad absorption bands are present at approximately 6.9, 7.15, 7.65, 8, 8.55, 8.95, 9.2 to 9.25, and 9.5 μ.

Qualitative differences between albumin fractions were noted in the infrared analysis of these fractions. For example, Fig. 9 is the infrared spectrum of the albumin fraction of the serum in Fig. 5A. This demonstrates that the absorption band in the serum at 7.35 μ is due, at least in part, to a band at this wave length in the albumin spectrum. The absence of an upward deflection at 6.95 μ is also noted.

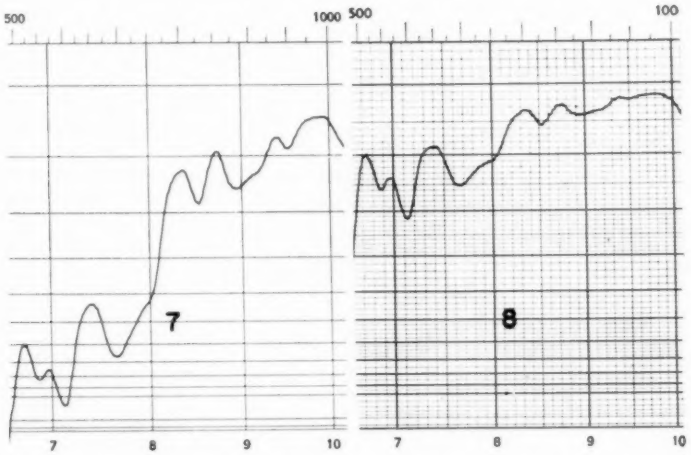


Fig. 7.—Infrared spectrum of pooled human albumin, scanned with water in the reference beam.

Fig. 8.—Infrared spectrum of the serum albumin fraction of a healthy adult, scanned with water in the reference beam.

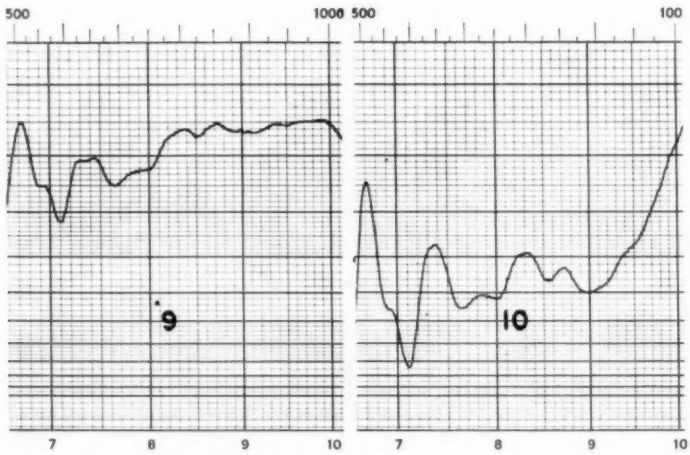


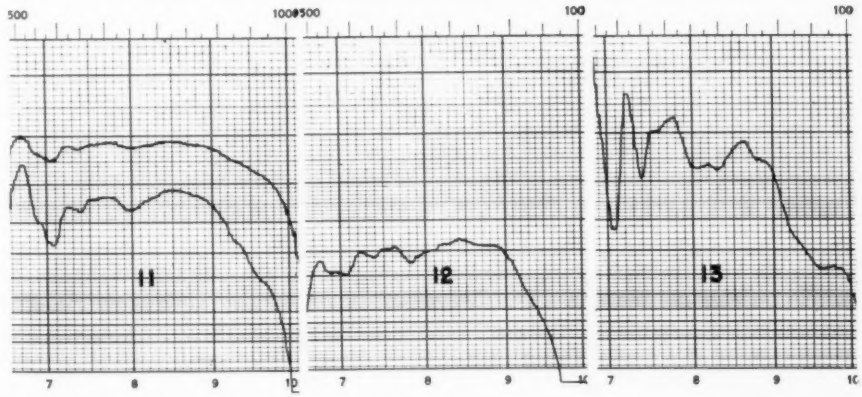
Fig. 9.—Infrared spectrum of the albumin fraction from the blood serum shown in Fig. 5A, scanned with water in the reference beam.

Fig. 10.—Infrared spectrum of the serum albumin from a patient with lymphosarcoma, scanned with water in the reference beam. The absorption at 8 μ is stronger in relation to the other bands than in the albumin fractions obtained from normal serum.

The pooled albumin sample of Fig. 7 was electrophoresed under standard operating conditions. The collected fractions were arbitrarily divided into three groups, designated as the slowest moving fraction, the middle fraction, and the fastest moving fraction. Although the three fractions obtained had very similar spectra, the middle fraction was observed to have no upward deflection at 6.95 μ (similar to Fig. 9), whereas the slowest moving fraction showed a very marked maximum at this wavelength.

To illustrate further that infrared spectroscopy can detect qualitative differences between albumin fractions, Fig. 10 is presented. This is the spectrum of the albumin fraction from a patient with lymphosarcoma. Again, no maximum is observed at 6.95 μ and the absorption at 8.0 μ is stronger in relation to the other bands than in albumin fractions obtained from normal serum.

Alpha globulin: The alpha and beta globulin fractions required considerably more concentration than the albumin and gamma globulin fractions before an infrared spectrum with more than one absorption band could be obtained. This indicates that in the spectra of normal sera they exert little influence upon the spectral pattern except for their absorption bands in the 7 μ region.



Figs. 11, 12, and 13.—Infrared spectra of alpha globulin fractions from three normal blood sera, scanned with water in the referance beam. Note the qualitative variations

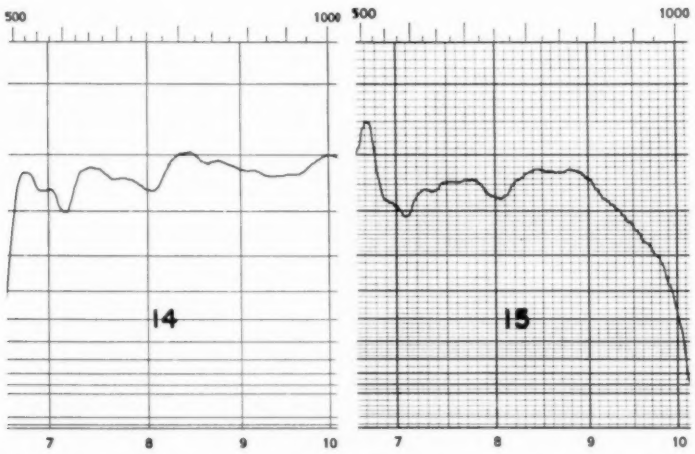
The infrared spectra of the normal alpha globulin fractions exhibited marked qualitative variations. Examples of three of the patterns observed are presented in Figs. 11, 12, and 13. In the lower tracing in Fig. 11, obtained by use of the pen expander, absorption bands are noted at 6.9, 7.05, 7.4, 7.9 to 8.0, 9.25, and 9.6 μ. Fig. 12 is similar to Fig. 11 except that it has a band at 7.82 μ instead of the broad band in the 8 μ region. In Fig. 13 bands at 8.3 and 8.8 μ are noted. Ratio differences between depths of bands was observed in these and the other nine spectra of alpha globulin fractions.

Beta globulin: The infrared spectra of two beta globulin fractions are shown in Figs. 14 and 15. In Fig. 14 absorption bands are present at 6.9, 7.15,

7.65, 7.9 to 8.1, 8.6, 9 to 9.1, and 9.3 to 9.7 μ. The spectrum in Fig. 15 has an additional band at 7.35 to 7.4 μ. This beta globulin fraction was separated from the serum of Fig. 5A. Thus, both the albumin and beta globulin fraction absorption bands in this area contribute to the band noted at 7.4 μ in the spectrum of unfractionated sera.

Four of the beta globulin fractions failed to have an absorption band at 8 μ. Five fractions had only small bands visible at 8 μ.

Gamma globulin: A representative spectrum of gamma globulin is shown in Fig. 16. A strong characteristic absorption is observed at 8.05 μ, in addition to the bands at 6.9, 7.15, and 8.65 μ. These bands were found in all twelve normal fractions. In addition, three of the fractions had an absorption band at 7.35 to 7.4 μ, one had a weak band at 7.6 μ, and the fraction in Fig. 16 shows a band at 9.3 μ.



Figs. 14 and 15.—Infrared spectra of beta globulin fractions from two normal blood sera, scanned with water in the reference beam. Fig. 15 is the fraction from the serum shown in Fig. 5A, and has an additional band at 7.35 to 7.4 μ.

Fig. 17 is a differential spectrum between two gamma globulin fractions having essentially identical individual spectra. Since the deep bands at 8.05 μ were cancelled out, the two samples were considered to be approximately equal in concentration. A prominent band at 7.25 μ was uncovered by this differential technique, and represents a real difference between the two fractions analyzed.

In the article devoted to the technique of obtaining infrared spectra of water solutions,[1] a spectrum of normal gamma globulin obtained commercially was presented which featured a very prominent 7.5 μ band. Not one of the twelve normal gamma globlin fractions in this series had a band at 7.5 μ although several showed a very weak absorption at 7.4 μ. This suggests that the commerical firm's method of separation results in a different form of gamma globulin or that it contains an impurity not present in this series.

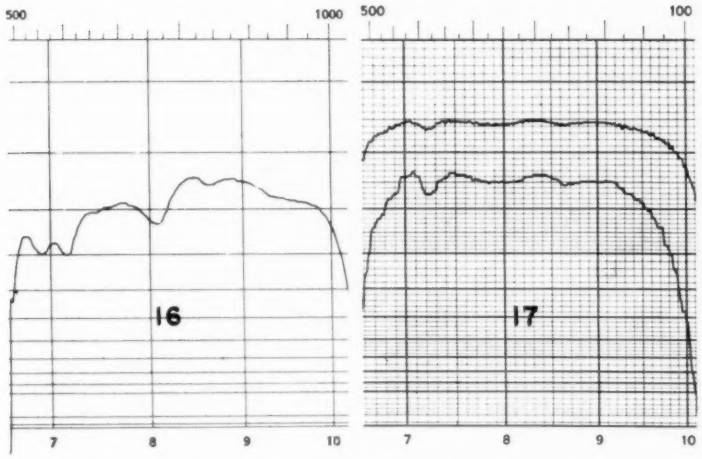


Fig. 16.—Infrared spectrum of the gamma globulin obtained from normal blood serum, scanned with water in the reference beam.

Fig. 17.—Differential scan between two normal gamma globulin fractions. The deep bands at 8.05 μ are cancelled out, and a prominent band at 7.25 μ is noted to represent a real difference between the two fractions.

Infrared Analysis of Blood Serum Samples Concentrated Fivefold by Evaporation.—Seventeen of the serum samples were concentrated with the expectation of increasing the sensitivity to minor variations in the chemical makeup. Surprisingly, no new absorption bands were observed when the concentrated sera were scanned either with water or concentrated pooled serum in the reference beam. Concentrating serum gives rise to problems in matching the concentration and salinity of the sample and reference solutions; but this disadvantage may be offset by the advantages of using thinner cells so that less light is lost to water absorption. Sensitivity to the abnormalities found in pathologic sera should be increased if concentration techniques can be made sufficiently reproducible.

DISCUSSION

The range of variation in the infrared spectra of normal blood serum is surprisingly small. Ninety per cent of the sera in this series had virtually identical spectra. In the other 10 per cent, the spectral variations occurred only in the region between 7.0 to 7.5 μ, with the single exception of the serum shown in Fig. 4 which had an absorptive band at 7.8 μ. Therefore, any variation in a serum pattern in the region between 7.5 and 10 μ probably represents a significant abnormality. A thorough investigation of this method for use as a diagnostic tool is now merited.

The most important finding in this study is the demonstration that infrared analysis of the individual protein fractions provides a reliable method for detecting major qualitative differences between two fractions which have identical electrophoretic properties. That there does exist a range of qualitative differences in the serum albumin and globulin fractions in a group of healthy young adults is most interesting.

The study of the qualitative differences in the serum protein fractions in various disease states could prove to be of diagnostic significance. In two spectra of gamma globulin fractions from sera of patients with multiple myeloma featuring high levels of gamma globulin[1] a strong 7.6 μ absorption band was noted. This band indicates a difference in either structure or composition from normal gamma globulin. It is hoped that an interpretation of such bands may provide a clue to actual structural differences between these molecules. Thus, the infrared analysis of the serum protein fractions adds a new dimension to the study of serum protein in health and in disease.

SUMMARY

The results of the infrared analysis of the serum protein from 105 healthy adults are presented. The range of variation in the infrared spectra of normal blood serum is small with most variations occurring in the region between 7.0 to 7.5 μ. Any variation in the region between 7.5 and 10 μ probably represents a significant abnormality.

The infrared analysis of the individual serum protein fractions demonstrated that a range of qualitative differences in the serum albumin and globulin fractions in the healthy adult group does exist, and so adds a new qualitative dimension to the investigation of the serum proteins.

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EFFECT OF LONG-TERM TREATMENT WITH CHLOROTHIAZIDE ON BODY FLUIDS, SERUM ELECTROLYTES, AND EXCHANGEABLE SODIUM IN HYPERTENSIVE PATIENTS

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The effects of chlorothiazide on body fluids and electrolytes have been studied in fifteen hypertensive patients who were taking the drug for 26 to 60 days.

No significant changes were found in blood volume, extracellular space, and total exchangeable sodium. Total body water and serum potassium were decreased.

The implications of these findings on our understanding of the antihypertensive mechanism of chlorothiazide are discussed.

THE administration of chlorothiazide to human subjects produces initially an increase in urine volume and a fall in body weight. Associated with these changes, the blood pressure is frequently reduced in hypertensive patients.[1, 2] In the first week of treatment, it has been shown that a reduction in blood volume[3] and sodium loss[4] accompany the decline in blood pressure.

The purpose of this report is to present studies on body fluid compartments, serum electrolytes, and on exchangeable sodium after more prolonged periods of treatment and to examine whether changes in any of these variables have a relation to the changes in blood pressure.

MATERIALS AND METHODS

The 15 patients chosen for this study had an average diastolic blood pressure of at least 100 mm. Hg as determined by daily home blood pressure readings with the patient in the recumbent position for a 2 week period.

The studies to be described were performed with the patients in the fasting state and in the recumbent position prior to and after treatment with 500 mg. of chlorothiazide† twice daily for 26 to 60 days. The patients were asked to avoid adding sodium chloride to the food but their diet was otherwise unrestricted. No potassium supplement was given. The posttreatment blood pressure was taken as the average of daily readings for the week prior to repeating the test.

In 4 patients total body water, extracellular fluid volume, plasma volume, serum sodium, serum potassium, and exchangeable sodium were measured. In 5 patients the same program was initiated but for various reasons it was not possible to obtain the complete series of tests before and after therapy. In an additional 6 subjects changes in blood volume alone were measured.

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†Supplied by Dr. John Beem, Merck Sharp & Dohme, Philadelphia, Pa.

Total body water was measured by the antipyrine dilution method of Brodie.[5] After starting an infusion of 5 per cent glucose in the left antecubital vein, 15 ml. of a 10 per cent antipyrine solution was administered over a 5 minute period through a syringe connected to a three way stopcock inserted into the intravenous set. The empty syringe was flushed out several times with the glucose solution and the infusion was discontinued. Blood samples were drawn from the right antecubital vein each hour between the third and sixth hours after the injection and the plasma antipyrine concentration was determined by the absorption at 350 mμ of nitrosoantipyrine obtained after addition of sulfuric acid and sodium nitrate to the deproteinized blood. The plasma concentration at zero time was obtained by extrapolation of the time-concentration curve back to the time of injection.

The blood volume was then estimated by the dye dilution method using Evans Blue.[6] A weighed volume of 3 to 5 ml. of a 5 per cent Evans Blue solution, was injected intravenously after the last blood sample was drawn for the previous test. The needle was flushed with 5 ml. of saline after the injection. After 15 minutes had elapsed for intravascular mixing, four samples of blood were withdrawn without venous congestion at 5 minute intervals from an indwelling needle in the antecubital vein of the opposite arm. Hematocrit determinations were made on the second and fourth samples of blood. The concentration of Evans Blue in the plasma was measured photometrically using a Beckman spectrophotometer at a wavelength of 620 mμ. The dilution of Evans Blue in plasma was estimated by extrapolation to the injection time of the time-concentration curve. In 2 experiments determinations in the same subject on 2 successive days gave values within 1 per cent of each other.

The extracellular fluid space was measured by the sodium dilution method of Kaltreider and collaborators.[7] The indicator, 3 μc of Na^{22}, was injected into the antecubital vein and blood samples were taken 20, 30, and 40 minutes afterward for determination of the radioactive sodium concentration. The extracellular fluid volume was determined from the radioactive sodium concentration at zero time as obtained by extrapolation of the time-concentration curve. The total exchangeable sodium was calculated from the Na^{22} concentration in serum after equilibration for 24 hours with allowance being made for the measured urinary excretion over this time. The formula was:

$$\text{Exchangeable Sodium}_{(Na_e)} = (Na^{22} \text{ injected} - Na^{22} \text{ excreted}) \frac{Na^{22}}{Na^{23}}$$

To supplement these detailed studies the blood pressure and changes in body weight of 83 patients on chlorothiazide therapy for an average of 5.4 months have also been recorded.

RESULTS

Body Weight.—The early reduction in body weight which usually appeared between 24 and 48 hours after chlorothiazide therapy persisted throughout the period of observation. In the group of 83 patients this change in weight averaged 3 per cent of the initial value (Fig. 1). This applied to those in whom the fall in systolic and diastolic pressure exceeded 10 per cent of the initial level (responders) and to those who did not show this change (nonresponders). In the 15 patients undergoing fluid volume studies the average fall in body weight was 2.1 kilograms regardless of the presence or absence of reduction in blood pressure.

Body Fluids, Serum Electrolytes, and Exchangeable Sodium.—Plasma volume determinations in 11 patients are shown in Table I. They are compared with the blood pressure changes which averaged −24/−13 mm. Hg, whereas the plasma volume changes averaged 51 ml., or less than a 2 per cent reduction. The observations on extracellular fluid volume, total body water, serum

TABLE I. RESULTS OF TREATMENT WITH CHLOROTHIAZIDE ON BLOOD PRESSURE, BLOOD VOLUME, AND HEMATOCRIT IN HYPERTENSIVE PATIENTS

PATIENT	DURATION OF TREATMENT (DAYS)	BLOOD PRESSURE* (MM. Hg)		PLASMA VOLUME (ML.)		HEMATOCRIT (%)	
		BEFORE	CHANGE	BEFORE	CHANGE	BEFORE	CHANGE
1	39	170 126	−12 −28	2,807	+253	47.8	−1.0
2	30	220 120	−68 −22	2,500	−243	49.4	+0.3
3	28	183 128	−32 −22	2,001	−151	54.9	+1.0
4	60	180 104	−30 −14	1,921	−318	43.7	+2.9
5	59	159 100	−26 −13	2,100	− 95	43.5	+0.4
6	29	160 100	−20 −13	2,950	− 30	42.9	−0.5
7	29	162 102	−12 −12	3,029	−279	47.5	−0.1
8	35	211 108	−22 −12	2,450	−130	37.4	+1.9
9	30	179 100	−27 − 8	3,164	+279	47.6	−2.4
10	26	166 101	−16 − 4	3,263	+ 8	45.7	−0.2
11	32	164 103	+ 2 + 1	2,060	+141	44.1	−0.4
Mean	36	177 108	−24 −13	2,568	− 51	45.9	+1.1

*Average of the recumbent blood pressure readings. Patients are arranged in order of decreasing effect of chlorothiazide on blood pressure.

electrolytes, and exchangeable sodium are summarized in Table II. Findings on 4 patients, in whom the complete set of observations were obtained, are illustrated graphically in Fig. 2.

The loss in body weight was consistently associated with a decline in total body water, which averaged 1.8 L. In one case neither a decline in body water, nor weight was observed (Patient 7, Table I). Extracellular fluid and plasma volume changes were insignificant, averaging −0.2 L. and −51 ml., respectively. It must therefore be concluded that the loss in water and body weight was the result of a fall in intracellular fluid and this was seen in the patients in whom estimations of both body water and extracellular fluid were made (Table III). In 4 there was an average fall of 1.9 L. and in one patient there was an increase in intracellular water and body weight.

The serum sodium concentration was in general unchanged, whereas the serum potassium showed an over-all downward trend, the average reduction being 0.4 mEq. per liter.

TABLE II. RESULTS OF TREATMENT WITH CHLOROTHIAZIDE ON BODY WEIGHT, SERUM

PATIENT	DURATION OF TREATMENT (DAYS)	BLOOD PRESSRE (MM. Hg) BEFORE	CHANGE	BODY WEIGHT (Kg.) BEFORE	CHANGE	PLASMA VOL. (ML.) BEFORE	CHANGE	HEMATOCRIT (%) BEFORE	CHANG
1	39	170 126	−12 −28	79.2	−2	2,807	+253	47.8	−1.0
2	70	220 120	−68 −22	83.4	−3.4	2,500	−243	49.4	+0.3
3	28	183 128	−32 −22	56.3	−1.9	2,001	−151	54.9	+1.0
4	30	194 128	−32 −20	89.5	−1.4				
5	56	166 110	−48 −19	64	−0.8				
6	27	172 106	−27 −11	84.6	−2.6				
7	31	190 130	0 −5	91.6	+1.8				
8	26	166 101	−16 −4	86.5	−0.8	3,263	+8	45.7	−0.2
9	32	164 103	+2 +1	59.4	−1	2,406	+141	44.1	−0.4

In agreement with others[8-10] we found that chlorothiazide led to variable effects on the exchangeable sodium. In 4 patients there was a decrease of 47 to 110 mEq. and in 3 patients there was a definite increase of 116 to 177 mEq.

DISCUSSION

Our finding that blood volume is not reduced after treatment with chlorothiazide is not in agreement with the data of Freis[8] and Dustan[9] who found a reduction in plasma volume of nearly 15 per cent as compared to our 2 per cent change. A partial explanation for this difference resides in the fact that some of their studies were performed on patients who were treated for a period of less than 8 days. In studies performed in our laboratories an early reduction in plasma volume was also found.[11] When the data obtained from patients treated for a period between 26 and 60 days is analyzed, however, this early reduction in plasma volume is no longer present. The tendency of the blood volume to increase after long-term therapy was noted by Freis[8] although he was unable to show a return to normal values. This difference between his and our results remains unexplained. Studies very similar to ours have recently been reported by Cottier[10] in which he showed that extracellular fluid volume was not reduced after 4 weeks of chlorothiazide therapy.

A change in the mechanism of the hypotensive action of chlorothiazide seems to occur with the passage of time. We have shown that cardiac output

ELECTROLYTES, BODY FLUIDS, AND EXCHANGEABLE SODIUM IN HYPERTENSIVE PATIENTS

EXTRACELLULAR VOLUME (L.)		TOTAL BODY WATER (L.)		SERUM Na (mEq./L.)		SERUM K (mEq./L.)		EXCHANGEABLE Na (mEq.)		EXCHANGEABLE Na (mEq./Kg.) BODY WEIGHT	
BEFORE	CHANGE	BEFORE	CHANGE	BEFORE	CHANGE	BEFORE	CHANGE	BEFORE	CHANGE	BEFORE	CHANGE
14.4	0	30.8	−1.6	143	0	4.5	−0.5	4,169	−47	52.7	+0.3
		36.6	−4.6	142	−2	4.2	−0.4				
9.0	+0.4	26.1	−2.3	140	0	4.2	0.3	2,013	−51	35.8	+0.2
				145	2	4.8	−0.2	3,862	−110	43.1	−0.5
				152	3	5.0	−1.1	2,610	+177	40.1	+4.0
		44.6	−3.8	140	+3	4.4	−0.5				
18.2	+1.2	34.8	+3.9	143	−3	4.3	0	3,164	+147	34.5	+0.9
18.0	−0.5	35.2	−2.2	143	+1	4.9	0	3,243	82	37.5	−0.6
11.2	+0.2	23.1	−2.3	140	0	4.3	−0.7	2,079	+116	35	+2.6

TABLE III. CHANGES IN BLOOD PRESSURE, BODY WEIGHT, AND INTRACELLULAR WATER IN HYPERTENSIVE PATIENTS AFTER TREATMENT WITH CHLOROTHIAZIDE

PATIENT	DURATION OF TREATMENT (DAYS)	BLOOD PRESSURE (MM. Hg)		BODY WEIGHT (Kg.)		INTRACELLULAR WATER* (L.)	
		BEFORE	CHANGE	BEFORE	CHANGE	BEFORE	CHANGE
1	28	183 128	−32 −22	56.3	−1.9	17.1	−2.7
2	26	166 101	−16 −4	86.5	−0.8	17.2	−1.7
3	32	164 103	+2 +1	59.4	−1	11.9	−1.5
4	39	170 126	−12 −28	79.2	−2	14.8	−1.6
5	31	190 130	0 −9	91.6	+1.8	16.6	+2.3

*The value obtained for Intracellular Water is the difference between total body water and extracellular volume.

fall at the time when plasma volume is reduced and rises again after a more prolonged period of therapy.[11] Total peripheral resistance is reduced toward normal at about the time that plasma volume is restored.

In a further attempt to determine the factor responsible for this hemodynamic change, we have investigated the relationship between body fluids and

exchangeable sodium after long-term therapy with chlorothiazide. Although the drug is a potent saluretic agent our results do not support the concept that sodium depletion is present after prolonged therapy, since we are unable to find a consistent reduction in total exchangeable sodium or in serum sodium after long periods of treatment. It is indeed doubtful whether sodium depletion plays an important role in the early phase of treatment since Hollander[4] was able to show that the abolition of a negative sodium balance by means of fluorohydrocortisone soon after initiation of treatment did not reverse the existing blood pressure reduction. However, the restoration of sodium balance by adrenal steroids does not guarantee the proper distribution of electrolytes. These

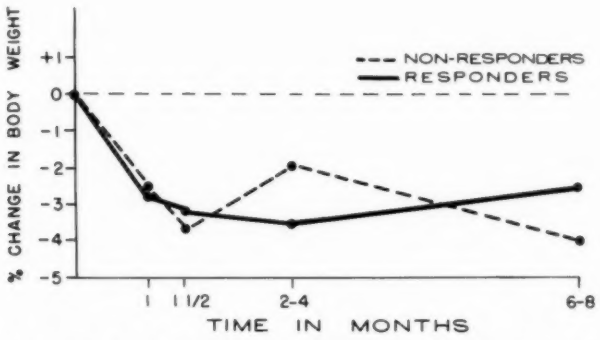

Fig. 1.—Change in body weight after administration of chorothiazide. *Responders* were taken as those patients who had a fall in systolic and diastolic pressure exceeding 10 per cent of the initial level.

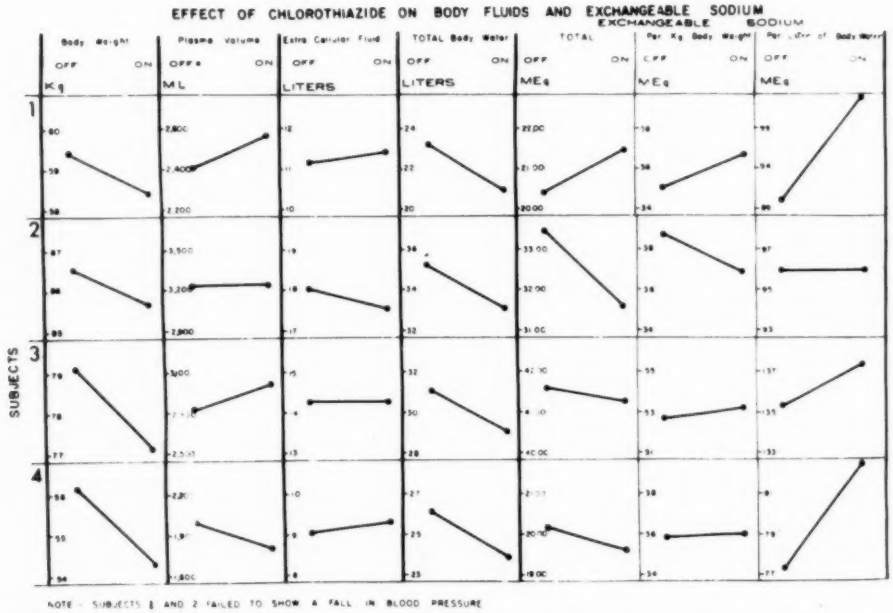

Fig. 2.—Effect of long-term therapy with chlorothiazide on body weight, plasma volume, extracellular fluid volume, total body water, and exchangeable sodium in 4 patients.

objections apply also to our results on exchangeable sodium since this technique can only estimate the pools of metabolically active ions and not their distribution or content in the body.

Almost every patient showed a fall in body weight. This was reflected by a decline in total body water. Since there was no change in extracellular fluid or plasma volume at this time, it was concluded that the fluid was removed from the intracellular compartment.

Since long-term therapy leads to a reduction in total peripheral resistance, it is possible that intracellular dehydration affecting the arteriolar wall may be of importance in the blood pressure reduction produced by chlorothiazide, and in this connection one might be tempted to suppose with Tobian[12] that dehydration of the vascular wall could bring about this change. However, the possibility of changes in the behavior of the muscular elements in the arteriolar wall will also require consideration.

Although the reduction in intracellular water after chlorothiazide may have been directly induced by the drug, the reservation must be made that it could be a secondary effect of some other change known to dehydrate cells. Potassium depletion could be responsible for this since it is known to occur regularly during chlorothiazide therapy[3, 9] and has been shown to result in intracellular depletion of water in normal individuals.[13] It has also been reported that low potassium diets may lower the blood pressure in experimental animals[14] and in man.[15] This explanation of the antihypertensive effect of chlorothiazide seems unlikely since the reduction in body potassium does not correspond with our observed changes in body water. This point requires further investigation.

Our observations on body fluids and electrolytes after long-term therapy with chlorothiazide show that a reduction in intracellular fluid volume is the only change which occurs regularly. It is possible that cellular dehydration by its effect on the blood vessels leads to a reduction in blood pressure.

SUMMARY

Changes in body fluid, serum electrolytes, and exchangeable sodium have been determined in hypertensive patients taking 1 Gm. of chlorothiazide daily for a period between 26 and 60 days. The most consistent change was a decrease in total body water which was believed to come from intracellular water depletion. No consistent changes were seen in plasma or extracellular fluid volume or exchangeable sodium. The importance of this finding in the blood pressure reducing action of chlorothiazide is discussed.

We wish to thank Dr. S. W. Hoobler for his valuable help in this project, and also Ray Warzynski for the chemical estimations, and Gladys Knoll for the technical assistance involved in this study.

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HEMODYNAMIC EFFECTS OF β METHYL 2,6-DIMETHYLPHENYL-ETHER OF CHOLINE (SKF 6890) ON THE SYSTEMIC AND CORONARY CIRCULATIONS

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Investigation of the hemodynamic effects of an agent which has been shown to block release of catecholamines from the adrenergic nerve endings (SKF 6890) is of interest in its own right, but also since it should help clarify the effects of the ganglion blocking drugs. Administration of SKF 6890 produced a decrease in cardiac output and stroke volume accompanied by reduced systemic arterial, pulmonary arterial, and right atrial blood pressures. Coronary blood flow was reduced but myocardial oxygen consumption was maintained and calculated cardiac efficiency declined. These effects are very similar to those of ganglion blocking drugs.

THE beta methyl form of 2,6-dimethylphenylether of choline, a quaternary nitrogen compound (Fig. 1), has been produced* under the code number of SKF 6890. The synthesis of this drug followed on the description of the interesting properties of TM-10 (SKF 5377: C,2,6-dimethylphenylether of choline).[1] Drugs of this group are believed, on excellent pharmacologic evidence, to be active because they prevent the release of catecholamines from the nerve endings of adrenergic nerves.[2] Although there are pharmacologic data concerning this aspect of the group of drugs,[1, 2] hemodynamic data are inadequate. Furthermore, investigation of the hemodynamic effects of a compound which blocks only the sympathetic outflow may cast further light upon the hemodynamic action of ganglion blocking drugs, which block both the sympathetic and parasympathetic systems.

MATERIAL AND METHODS

This study was carried out in 10 mongrel dogs, which weighed between 16 and 24 kilograms, average 20.3 kilograms. Anesthesia was secured by administration of 3 mg. per kilogram of morphine sulfate subcutaneously followed 1 hour later by 0.25 ml. per kilogram of a 50:50 mixture of veterinary Nembutal (60 mg. per milliliter) and Dial-urethane.†

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*Smith Kline & French Laboratories, Philadelphia, Pa.

†Supplied by Ciba Pharmaceutical Products Inc., Summit, N. J. (Contains Dial 100 mg. per milliliter, monethylurea 400 mg. per milliliter, and urethane 400 mg. per milliliter.)

During the ensuing hour, cardiac catheters were placed in the pulmonary artery, coronary sinus, and right atrium and an indwelling needle was placed in the femoral artery. Complete air collection was ensured by placement of a cuffed endotracheal tube connected through a non-rebreathing valve to a Tissot spirometer and to a three way stopcock which permitted a rapid change from inhalation of room air to inhalation of a mixture of 21 per cent oxygen, 64 per cent nitrogen, and 15 per cent nitrous oxide. Cardiac output was determined by the Fick principle and coronary blood flow by the nitrous oxide saturation method. Pressures were measured by Statham strain gauges with the mean pressure determined by electrical integration on the Sanborn Poly-Viso. Cardiac rate was measured from an appropriate electrocardiographic lead. Methods and calculations have been previously described.[3]

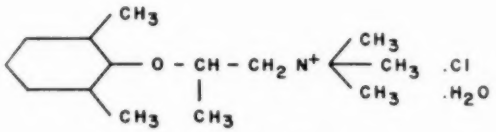

Fig. 1.—SKF 6890.

After the control hemodynamic measurements, the drug was given intravenously in a dose of 5 mg. per kilogram to all dogs except one which received 10 mg. per kilogram. The drug was administered slowly because it was established by preliminary experiments that if it was given rapidly a transitory rise in blood pressure and heart rate would occur followed by a transitory decrease in pressure. If the drug was administered slowly over a period of 5 to 10 minutes a rate of administration could be found in which there was a steady decline in blood pressure without the transitory overshoot and with which no marked change occurred in cardiac rate. Tachyphylaxis occurred, even over a period of 5 to 10 minutes, so that the rate of administration was gradually increased to secure the hypotensive effect. The second set of hemodynamic observations were begun an average of 18 minutes after the onset of drug administration when the animals' blood pressure, pulse, and respiration seemed stable.

RESULTS

Results, summarized in Table I, reveal that after administration of SKF 6890 there was a slight and insignificant acceleration in pulse, whereas systemic arterial blood pressure ($p<0.02$), mean pulmonary arterial blood pressure ($p<0.05$) and right atrial mean pressure ($p<0.05$) decreased significantly. Neither oxygen consumption of the animals nor the body respiratory quotient changed significantly. The systemic arteriovenous oxygen difference ($p<0.01$) and the venous-arterial difference for carbon dioxide ($p<0.02$) increased significantly. The coronary sinus oxygen content decreased significantly ($p<0.01$) and there was significant widening of the arteriocoronary sinus oxygen difference ($p<0.001$). Neither the hemoglobin nor hematocrit changed significantly during the experimental period.

Cardiac output was slightly but significantly reduced ($p<0.05$) and the stroke volume was even more significantly reduced ($p<0.01$). Total peripheral resistance increased slightly but insignificantly, with considerable variation from animal to animal. Total pulmonary resistance was similarly variable. Left ventricular work ($p<0.01$) and right ventricular work ($p<0.01$) were significantly reduced.

TABLE 1

DETERMINATION	BEFORE (± S.D.*)	AFTER (± S.D.)	% CHANGE	P VALUE
Cardiac rate	82 ± 27	91 ± 35	+10.1	<0.1
Systemic mean arterial blood pressure (mm. Hg)	128 ± 9	118 ± 10	- 7.3	<0.02
Pulmonary mean arterial blood pressure (mm. Hg)	16 ± 5	14 ± 4	-13.7	<0.05
Right atrial mean blood pressure (mm. Hg)	4.0 ± 1.3	3.2 ± 1.6	-20.0	<0.05
Body O_2 consumption (ml./min.)	119 ± 32	122 ± 32	+ 2.9	<0.2
Body respiratory quotient	0.85 ± 0.07	0.88 ± 0.09	+ 3.5	<0.1
Arterial O_2 content (ml./100 ml. blood)	18.1 ± 2.0	18.3 ± 2.1	+ 1.2	<0.5
Arteriovenous O_2 difference (ml./100 ml. blood)	4.8 ± 0.5	5.6 ± 1.1	+16.7	<0.01
Mixed venous arterial CO_2 difference (ml./100 ml. blood)	4.0 ± 0.8	4.8 ± 1.5	+20.0	<0.02
Coronary sinus blood O_2 content (ml./100 ml. blood)	4.8 ± 1.8	3.2 ± 1.0	-31.3	<0.01
Arteriocoronary sinus O_2 difference (ml./100 ml. blood)	13.3 ± 1.9	15.4 ± 2.1	+15.8	<0.001
Hemoglobin content arterial blood (Gm./100 ml.)	15.0 ± 1.8	15.1 ± 1.9	+ 0.7	<0.6
Hematocrit (arterial blood)	48 ± 6	49 ± 6	+ 2.1	<0.2
Cardiac output (L./min.)	2.6 ± 0.8	2.3 ± 0.8	-10.4	<0.05
Stroke volume (ml.)	33 ± 11	27 ± 10	-15.1	<0.01
Total peripheral resistance (dynes cm.$^{-5}$/sec.)	4,348 ± 1,224	4,578 ± 1,479	+ 5.3	<0.4
Total pulmonary resistance (dynes cm.$^{-5}$/sec.)	553 ± 250	530 ± 175	- 4.2	<0.7
Left ventricular work (Kg. M./min.)	4.4 ± 1.6	3.7 ± 1.4	-18.2	<0.01
Right ventricular work (Kg. M./min.)	0.6 ± 0.2	0.4 ± 0.2	-21.8	<0.01
Coronary blood flow (ml./100 Gm./min.)	73 ± 15	61 ± 13	-16.5	<0.001
Myocardial O_2 consumption (ml./100 Gm./min.)	9.7 ± 2.0	9.3 ± 2.4	- 4.1	<0.4
Coronary vascular resistance (units)	1.80 ± 0.36	2.04 ± 0.53	+13.3	<0.05
Index of efficiency (left ventricular work/cardiac metabolic rate O_2)	0.47 ± 0.16	0.41 ± 0.15	-12.8	<0.05

*S. D. = standard deviation of the mean.

The coronary blood flow decreased ($p<0.01$), but the cardiac metabolic rate for oxygen was maintained without significant change and the cardiac respiratory quotient did not change significantly. Coronary vascular resistance increased ($p<0.05$) and cardiac efficiency decreased ($p<0.05$).

DISCUSSION

It is of considerable interest that the hemodynamic effects of SKF 6890 are so similar to the hemodynamic effects of the well-known ganglionic blocking agents, hexamethonium[3, 4] and mecamylamine.[5] It has been suggested that the decreased cardiac output produced by hexamethonium is effected through decreased force of myocardial contraction secondary to lowered arterial pressure and decreased sympathetic discharge,[6] especially since hexamethonium has direct inotropic action on the papillary muscle of the cat and the hypodynamic dog heart-lung preparation.[7] The present data are certainly compatible with this suggestion. Furthermore, they are compatible with the recent hemodynamic data linking the inotropic effects of exercise to the cardiac adrenergic nerve supply.[8] In the present series of experiments, as with the ganglion blocking agents,[5] peripheral vascular resistance has actually remained the same or increased somewhat rather than decreased.

SUMMARY

1. The hemodynamic effects of the beta methyl form of 2,6-dimethylphenyl-ether of choline (SKF 6890) have been studied.

2. Administration of the drug is associated with a decrease in cardiac output and stroke volume accompanied by reduced systemic arterial, pulmonary arterial, and right atrial blood pressures.

3. Coronary blood flow is significantly reduced but myocardial oxygen consumption is maintained in spite of reduced left ventricular work, and cardiac efficiency declines.

4. These hemodynamic effects are similar to those of the ganglion blocking agents hexamethonium, mecamylamine, and, so far as is known, pentolinium.

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COMPARISON OF DITHIAZANINE IODIDE AND PYRVINIUM PAMOATE IN THE TREATMENT OF ENTEROBIASIS (PINWORMS)

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Forty-eight children and six adults infected with pinworms were treated with dithiazanine iodide or pyrvinium pamoate.† Many of those treated with dithiazanine iodide vomited and did not complete the course of treatment. In those who completed treatment, eradication of the pinworm was the rule.*

In a series of 29 individuals given a single dose of pyrvinium pamoate, vomiting occurred in only one. The treatment eliminated the pinworms in the remaining 28 patients.

INFECTIONS caused by the parasite *Enterobius vermicularis* (pinworm) are common, particularly in children. Often the infections are light and cause no symptoms. However, in children, early infections tend to be heavy and associated with annoying rectal itching, particularly at night. Mothers of these children are often disturbed and they seek a simple and effective treatment. Many drugs have been tried in the past with varying degrees of success and frequent side effects.

Gentian violet is effective but it stains the clothing and acts slowly. Hexylresorcinol is not very active and it often leads to abdominal cramps. Terramycin (oxytetracycline) is effective but several doses must be given and it is rather expensive. One of the most satisfactory drugs for pinworms has been piperazine citrate. Repeated doses are required and vomiting may occur. With all these drugs relapses are frequent unless every member of an infected group is treated.

Dithiazanine iodide, a blue cyanine compound, was shown to have anthelmintic activity by McCowen and others.[1] It was effective against canine ascarides, hookworm, and whipworm. Dithiazanine iodide was also observed to be active against murine pinworms.[1, 4] Recently there have been reports on the use of the drug against various human parasites.[2, 3] The drug appears to be particularly useful for infections with *Strongyloides stercoralis, Ascaris lumbricoides,* and whipworm but rather ineffective with hookworm. Miller and his group[1] treated 35 patients with pinworms and all appeared to be cured. The children were given 100 mg. enteric-coated tablets three times daily for five days and vomiting occurred in 5 of 31.

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*Supplied by Eli Lilly & Company as Delvex.

†Provided by Parke, Davis & Company as Povan.

Pyrvinium chloride, a red cyanine dye, was found by Weston and associates[5] to have activity against pinworms in laboratory animals. Later, the drug was shown to be nearly 100 per cent effective against pinworm infections in humans.[6-9] A bitter taste, nausea, and vomiting were frequent. Beck and others[9] found that pyrvinium pamoate was well received and 94 per cent of 34 children were cured with multiple small doses. They also found that a single larger dose was well tolerated and effective in 96 per cent of 100 patients. The single dose regimen was as popular as it was effective.

In the present study dithiazanine iodide was given in multiple doses to 23 children who had pinworms and compared in effectiveness and side effects with a single dose of pyrvinium pamoate administered to 29 subjects.

MATERIAL AND METHODS

The patients treated were mainly children at the Shriners Hospital for Crippled Children, Minneapolis, Minnesota,* plus a few children and adults in families of hospital employees. The diagnosis was made by microscopic examination for the ova on a single cellophane tape anal swab taken in the morning.

Dithiazanine iodide was given either as plain or enteric coated tablets which contained 25 mg. of the drug. The daily dosage was 5 mg. per pound body weight, not to exceed 600 mg. The daily dosage was divided into 2 or 3 doses and given for 5 days. Cellophane tape studies were made for ova about one and three weeks after treatment.

Pyrvinium pamoate was given as a raspberry flavored syrup which contained 10 mg. of pyrvinium per milliliter. A single dose of 5 ml. per 20 pounds body weight was given before breakfast. Search for pinworm ova was made 7 and 21 days after treatment.

RESULTS

One hundred and nine children at the Shriners Hospital were examined and 38 were found infected with pinworms. In addition, 10 other children and 6 adults who had the parasite were treated.

Dithiazanine Iodide.—Uncoated tablets were given to 11 children ranging in age from 3 to 14 years and in weight from 33 to 98 pounds. Treatment was discontinued in 9 because of vomiting. In the 2 children who completed treatment no ova were found thereafter.

Enteric coated tablets were given to 12 children ranging from 4 to 13 years of age and from 32 to 96 pounds in weight. Four children vomited and treatment was discontinued. Vomiting usually occurred about 2 hours after the first dose and was not accompanied by nausea. Six children who completed the treatment and received full doses were all freed of pinworm ova. One child continued treatment in spite of vomiting once. In 2 children who vomited, the dosage was reduced by one half. There was no further vomiting and the pinworms were eliminated. The coated tablets were also given to 2 adults. One of the adults had no side effects and cure was effected. The other adult had vomiting and diarrhea. Therapy was discontinued after 3 days without disappearance of the pinworm ova.

*These patients were treated through the cooperation of Dr. Donald R. Lannin, Chief of Staff.

Pyrvinium Pamoate.—Twenty-five children and 4 adults were treated. One child vomited two hours after administration of the drug and treatment was not repeated. In no other case was there vomiting or discomfort. The two post-treatment examinations did not reveal pinworm ova in any of the 28 subjects who completed the treatment.

DISCUSSION

Dithiazanine iodide is an effective drug in the treatment of enterobiasis. In the doses generally recommended, however, vomiting was frequent in the children and treatment was discontinued in about one half the patients. This difficulty occurred both with the plain and enteric coated tablets. Miller and associates[4] reported fewer side effects with the drug; in one series of 26 children who were given a larger dose (200 mg. three times daily for 5 days) no side effects were recorded but in a second group of 31 children one half this dose led to 5 episodes of vomiting. Treatment can be completed by most patients even though vomiting and diarrhea may interrupt therapy or require a reduction in dosage. Miller found that vomiting occurred in 11 of 27 adults who were given dithiazanine iodide in a dose of 800 mg. per day.[4]

Pyrvinium pamoate given in a simple syrup was well tolerated by children. Vomiting ensued in only one of 29 patients and no diarrhea was noted after a single large dose. Red staining of the stools was frequent. A single dose of the drug was effective in eliminating pinworms and their ova. The drug appears to be especially useful for mass treatment in institutions and in families because of the low cost, ease of administration, and effectiveness.

SUMMARY

1. Dithiazanine iodide was given in multiple doses to 25 patients with enterobiasis (pinworms). Treatment was discontinued in 14 because of vomiting or diarrhea. Treatment was completed in 8 of 12 children who received enteric coated tablets and in only 2 of 11 children who were given uncoated tablets. Pinworm ova were not found in any of the patients who completed the treatment, either in full or half doses.

2. A single dose of pyrvinium pamoate was administered to 25 children and 4 adults with pinworms. There were no side effects except in one child who vomited once two hours after administration of the drug. The treatment eliminated pinworms in all but this one patient. The treatment is simple, safe, and effective. It is especially recommended for treatment of groups in institutions and families.

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SERUM LEUCINE AMINOPEPTIDASE ACTIVITY IN HEPATOBILIARY AND PANCREATIC DISEASE

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Serum leucine aminopeptidase (LAP) activity has been investigated in 121 patients with various disorders of the hepatobiliary system and pancreas, and in 218 control subjects, including normal adults, pregnant women, and patients with neoplastic and other diseases which do not affect the liver and pancreas. The findings have been compared with the results of other tests of hepatobiliary function which were carried out simultaneously.

Evidence is presented to show that, although the serum LAP level is a sensitive and reasonably specific index of hepatobiliary disease, it is of no value in differentiating between the various disorders that affect the liver, bile ducts, and pancreas, and adds no useful information not already provided by other tests of hepatobiliary function in common use.

It is concluded that the determination of serum LAP activity does not merit adoption as a diagnostic test in hepatobiliary or pancreatic disease.

IN 1957 Fleischer and co-workers[1] called attention to the increase in serum leucine aminopeptidase (LAP) activity that occurs in patients with hepatobiliary disease. More recently Rutenberg and associates[2] have reported that the serum LAP level is also raised in patients with carcinoma of the pancreas, even in the absence of jaundice, and suggested that the test may be of diagnostic value in that disease.

The present investigation was undertaken to evaluate further the significance of alterations in serum LAP activity in hepatobiliary and pancreatic disease.

MATERIALS

Serum LAP activity was measured in 530 samples of blood obtained from 330 individuals, including 121 patients with diseases (including neoplasms) in which the liver, biliary tract, or pancreas was affected, 62 normal healthy adults, 60 patients with a variety of diseases unaccompanied by clinical or laboratory evidence of hepatobiliary or pancreatic disease, 50 patients with neoplasms of nonpancreatic origin and without clinical or laboratory evidence of hepatic metastases, 29 normal pregnant women, and 14 patients with acute myocardial infarction accompanied by a significant rise in serum glutamic oxalacetic transaminase activity.

Parallel tests of liver function, carried out on a high proportion of the serum samples examined, included determinations of serum bilirubin, Bromsulphalein retention (45 minutes

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after an injection of 5 mg. of dye per kilogram), cephalin-cholesterol flocculation, thymol turbidity, serum alkaline phosphatase, and serum glutamic oxalacetic transaminase.

Cirrhosis.—Of the 25 patients investigated 23 had Laennec's cirrhosis and 2 had postnecrotic cirrhosis. All exhibited classical clinical and laboratory features of the disease, and in 19 the diagnosis was confirmed by needle biopsy of the liver.

Viral Hepatitis.—One patient had homologous serum hepatitis, and 11 patients had infectious hepatitis. All were seen early in the course of the disease and exhibited typical clinical and laboratory features; in 10 the diagnosis was confirmed by liver biopsy. In 4 of the patients the lesions demonstrated histologically were consistent with subacute hepatic necrosis.

Infectious Mononucleosis.—In all 4 patients studied the hematologic and clinical pictures were typical, and the absorbed heterophil antibody titer was 1:160 or higher. Although all showed functional abnormalities consistent with hepatocellular injury and inflammation, confirmed by liver biopsy in 3, only 2 were clinically jaundiced.

Toxic Hepatitis.—In all 5 patients studied the hepatitis was a complication of bacteremia of extrahepatic origin, and was accompanied by jaundice and laboratory evidence of hepatocellular injury. There was one case each associated with typhoid fever, paratyphoid fever, pneumococcal pneumonia, staphylococcal endocarditis, and staphylococcal septicemia.

Granulomatous Hepatitis.—Nine patients with sarcoidosis and 3 with tuberculosis with histologically demonstrable hepatic granulomata were investigated. All showed abnormalities of liver function, but only two were clinically jaundiced.

Obstructive Jaundice.—In the 18 patients studied, obstruction of the common bile duct was due to stone in 9 and to carcinoma of the pancreas in 9. The diagnosis of biliary obstruction was established by surgical exploration in each case, and that of pancreatic carcinoma by surgical biopsy. The presence of hepatic metastases was confirmed by direct inspection at the time of laparotomy and/or by needle biopsy of the liver. All patients were restudied after relief of the obstruction by appropriate surgical measures.

Gall Bladder Disease.—In all 13 patients the diagnosis of cholecystitis was confirmed by surgical exploration and histologic examination of the gall bladder. The process was acute in 7 and chronic in 8. Stones were demonstrated in the gall bladder in 6 of the former and all of the latter. Although none had choledocholithiasis, all of the acute and 4 of the 8 chronic cases had a mild obstructive jaundice presumably as a result of associated cholangitis.

Pancreatic Disease.—In addition to the 9 patients with pancreatic carcinoma noted previously, 6 patients with acute pancreatitis were studied. All exhibited typical clinical features and a significant increase in serum amylase and/or lipase. In 2 the disease was accompanied by a mild obstructive jaundice.

Neoplastic Disease.—The 86 patients with neoplastic disease included the following:

	Total number	Without hepatic metastases	With hepatic metastases
Carcinoma, breast	15	8	7
Carcinoma, colon	14	8	6
Carcinoma, lung	14	9	5
Carcinoma, pancreas	9	3	6
Carcinoma, prostate	7	7	0
Carcinoma, stomach	5	5	0
Carcinoma, cervix	3	2	1
Carcinoma, kidney	3	3	0
Melanoma	3	0	3
Carcinoma, rectum	2	2	0
Sarcoma	2	2	0
Glioblastoma	2	2	0

Carcinoma, primary site	1	0	1
Meningioma	1	1	0
Carcinoma, thyroid	1	1	0
Carcinoma, bladder	1	1	0
Carcinoma, ovary	1	1	0
Carcinoma, parotid	1	1	0
Carcinoma, duodenum	1	0	1
	86	56	30

In all cases the diagnosis was confirmed histologically.

METHODS

Serum LAP activity was measured by a previously described method[3] in which l-leucyl-β-naphthylamine hydrochloride serves as a chromogenic substrate from which β-naphthylamine is liberated by enzymatic hydrolysis. The freed β-naphthylamine is converted to a colored azo dye by a modification of the Bratton-Marshall reaction, and quantitated colorimetrically in terms of optical density at 560 mμ; an O.D. of 0.001 was considered one unit, and equivalent to 0.1 meg. of β-naphthylamine. The mean and standard deviation for 62 healthy adults was 252 ± 35 units. Values above 322 units were considered abnormal.

Serum glutamic oxalacetic transaminase activity was estimated by a modification of the Karmen method.[4] Other tests of liver function were carried out as described previously.[5]

RESULTS

Serum LAP Activity in Hepatobiliary and Other Diseases.—Table I summarizes the results of serum LAP determinations in the various disease groups investigated. To facilitate comparisons and to minimize inequalities introduced by differences in the number of serial determinations carried out in patients with each of these disorders, only the *peak values* for individual patients have been taken into consideration. Table II summarizes the changes in serum LAP activity observed in patients studied serially during the course of their illnesses.

Hepatocellular disease: All but 3 of the 46 patients with hepatocellular disease had elevated levels of serum LAP activity. The exceptions included 2 individuals with inactive cirrhosis and one with mild viral hepatitis who had a borderline value of 320 units.

Judging from the means and standard deviations, the levels attained in patients with cirrhosis, acute viral hepatitis, and infectious mononucleosis did not differ significantly. However, those in patients with toxic hepatitis associated with severe systemic infections were significantly lower than those in patients with cirrhosis ($t = 2.60$, $p < 0.02$) or acute viral hepatitis ($t = 3.31$, $p < 0.01$). Of interest is the fact that the serum LAP level was raised in 5 of the 6 patients with cirrhosis classified as inactive on the basis of their clinical, functional, and biopsy findings.

In 11 of the 20 patients with cirrhosis who were followed serially, serum LAP activity returned to a normal level. Although this coincided with improvement in the clinical and functional status of the liver, there was evidence of residual hepatic disease in all patients.

Four of the 11 patients with acute viral hepatitis achieved complete clinical and functional recovery with a fall in serum LAP activity to a normal level. High values persisted in 6 of the 7 remaining patients in whom the disease was still active. The exception was of particular interest in that the patient had

TABLE I. SERUM LAP ACTIVITY IN HEPATOBILIARY, PANCREATIC, NEOPLASTIC, AND OTHER DISEASES

CLINICAL STATUS	SUBJECTS	SERUM LAP ACTIVITY			
		DETERMINATIONS	MEAN ± S.D.	RANGE	% ABNORMAL
Normal adults	*62*	*62*	*252 ± 55*	*130-300*	*0*
Hepatocellular disease	*46*	*197*	*531 ± 194*	*189-990*	*93*
Cirrhosis	25	114	523 ± 204	189-990	92
Viral hepatitis	12	66	592 ± 187	320-900	92
Infectious mononucleosis	4	12	559 ± 170	330-740	100
Obstructive jaundice (Nonmet)	*12*	*32*	*692 ± 168*	*430-990*	*100*
Common duct stone	9	25	739 ± 154	480-990	100
Carcinoma of pancreas without hepatic metastases*	3	7	553 ± 129	430-730	100
Infiltrative disease	*42*	*56*	*586 ± 201*	*305-1,000*	*95*
Granulomatous hepatitis	12	14	622 ± 190	380-1,000	100
Hepatic metastases*	30	42	572 ± 202	305-1,000	93
Gall bladder disease					
Acute cholecystitis	7	8	642 ± 204	385-950	100
Chronic cholecystitis	8	11	301 ± 104	140-450	50
Pancreatic disease					
Acute pancreatitis	6	8	328 ± 79	195-455	67
Carcinoma of pancreas	*9*	*25*	*702 ± 187*	*430-1,000*	*100*
without hepatic metastases*	3	7	553 ± 129	430-730	100
with hepatic metastases*	6	18	777 ± 165	560-1,000	100
Neoplastic disease without hepatic metastases					
Carcinoma of pancreas*	3	7	553 ± 129	430-1,000	100
Nonpancreatic neoplasms	53	53	239 ± 55	125-345	8
Neoplastic disease with hepatic metastases	*30*	*42*	*572 ± 202*	*305-1,000*	*93*
Carcinoma of pancreas*	6	18	777 ± 165	560-1,000	100
Nonpancreatic neoplasms*	24	24	521 ± 176	305-990	79
Noncoplastic disease with no evidence of hepatic or pancreatic involvement	*60*	*60*	*210 ± 59*	*90-305*	*0*
Normal pregnancy					
2 months	5	5	308 ± 8	300-320	0
3-5 months	15	15	382 ± 54	325-520	100
At term	9	9	694 ± 174	420-950	100
Myocardial infarction with elevated serum glutamictransaminase	*14*	*14*	*230 ± 55*	*140-330*	*7*

For those subjects with multiple determinations only peak values are reported.
*These values have been entered under more than one category to facilitate comparisons.

massive hepatic necrosis with intense jaundice. During the first few days of her illness, serum LAP activity was greatly increased, but terminally, despite a rise in the serum bilirubin level to 50 mg. per cent and the development of hepatic coma, the serum LAP value fell to 235 units.

Obstructive jaundice: Obstructive jaundice invariably raised serum LAP activity to abnormal levels which were significantly higher than those found in patients with hepatocellular disease ($t = 2.76$, $p < 0.01$). Although the rise

was somewhat smaller in patients with pancreatic carcinoma uncomplicated by hepatic metastases than in those with common duct stone, the difference was not statistically significant.

In all 3 patients with pancreatic carcinoma and in 6 of the 9 with common duct stone, surgical relief of biliary obstruction led to a prompt fall in serum LAP activity to a normal level. This occurred within 3 to 5 days, and at a time when jaundice was still evident clinically.

Infiltrative disease of the liver: Serum LAP activity was abnormally high in all 12 patients with granulomatous hepatitis caused by sarcoidosis or tuberculosis, and in all but 2 of the 30 patients with metastatic neoplasms which involved the liver. The two exceptions had borderline values of 305 and 310 units.

TABLE II: EFFECT OF TREATMENT ON SERUM LAP ACTIVITY

DISEASE	NO. OF PATIENTS	INCREASED SERUM LAP (%)
Cirrhosis		
before treatment	20	100
after treatment	20	45
Viral hepatitis		
before treatment	11	100
after treatment		
inactive	4	0
active	7	86
Obstructive jaundice caused by cholelithiasis		
before treatment	9	100
after choledocholithotomy	9	34
Obstructive jaundice caused by carcinoma of pancreas without hepatic metastases		
before treatment	3	100
after cholecystojejunostomy	3	0
Obstructive jaundice caused by carcinoma of pancreas with hepatic metastases		
before treatment	6	100
after cholecystojejunostomy	6	100

The mean for the group as a whole fell between those for patients with hepatocellular disease and obstructive jaundice, but statistically did not differ significantly from either.

The rise in serum LAP activity occurred in the absence of jaundice in more than 66 per cent of this group.

Gall bladder disease: All 7 patients with acute cholecystitis had a significantly elevated serum LAP level. The mean for the group fell between those for patients with hepatocellular disease and obstructive jaundice, but statistically did not differ significantly from either. Although mild jaundice was present in every patient, and cholelithiasis in all but one, no evidence of biliary obstruction or common duct stone was found at the time of surgical exploration. Therefore, the jaundice was interpreted as a manifestation of complicating cholangitis.

In contrast, only 4 of the 8 patients with chronic cholecystitis exhibited abnormally high serum LAP values. Moreover, the mean for the group as a whole was significantly lower than that for the groups with hepatocellular disease ($t = 4.68$, $p < 0.01$) or obstructive jaundice ($t = 6.08$, $p < 0.01$). Only those with increased serum LAP activity exhibited jaundice. As in the group with acute cholecystitis, the latter appeared to be related to a complicating cholangitis rather than to biliary obstruction.

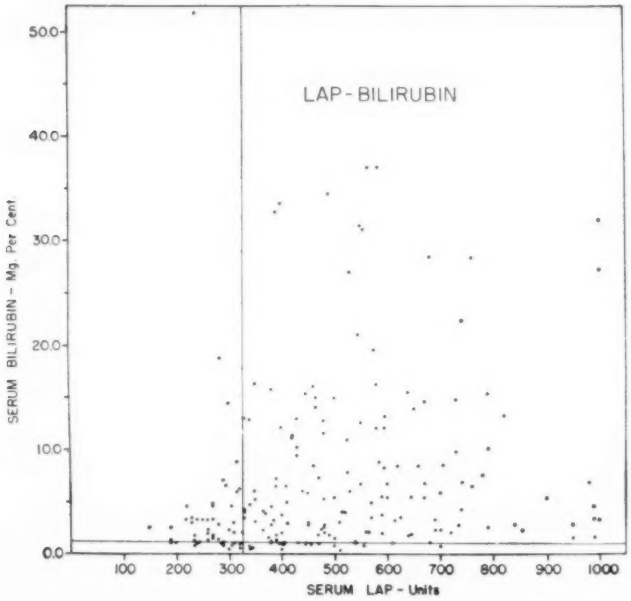

Fig. 1.—Relationship between serum LAP activity and bilirubin concentration in 210 serum samples. Horizontal and vertical lines indicate upper limits of normal for the two tests.

Pancreatitis: Serum LAP activity was increased in 4 of the 6 patients with acute pancreatitis, but none showed really high values. The mean for the group as a whole was significantly lower than that for the patients with acute cholecystitis ($t = 3.47$, $p < 0.01$), hepatocellular disease ($t = 4.42$, $p < 0.01$), or obstructive jaundice ($t = 5.89$, $p < 0.01$). Only 2 of those with elevated levels exhibited a mild, transient, obstructive jaundice of the type commonly seen in this disease.

Carcinoma of the pancreas: Without exception the 9 patients with carcinoma of the pancreas, all of whom had biliary obstruction, exhibited increased serum LAP activity comparable to that found in patients with obstructive jaundice caused by common duct stone. In those with hepatic metastases the levels were significantly higher than in those without hepatic metastases ($t = 2.81$, $p < 0.05$). In both groups they were significantly higher than in those found in patients with acute pancreatitis ($t = 5.50$, $p < 0.01$, and $t = 4.85$, $p < 0.01$, respectively).

After surgical relief of biliary obstruction, serum LAP activity reverted to normal in all 3 patients without hepatic metastases, but in none of the 6 with metastases. This effect was observed within the first week postoperatively, at a time when all patients showed a fall in serum bilirubin but were still clinically jaundiced.

Nonpancreatic neoplastic disease: Only one of the 53 patients *without* hepatic metastases in this group had an elevated serum LAP level. In contrast, the serum LAP level was abnormally raised in 79 per cent of the 24 patients *with* metastases, despite the absence of jaundice in most. Although many of the individual values were as high as those found in patients with pancreatic carcinoma with hepatic metastases, the mean for the group was significantly lower ($t = 3.11$, $p < 0.01$).

Noncoplastic disorders without evidence of hepatic or pancreatic disease: None of the 60 patients in this group, who had a wide variety of disabling diseases which necessitated hospitalization, had an abnormal serum LAP value.

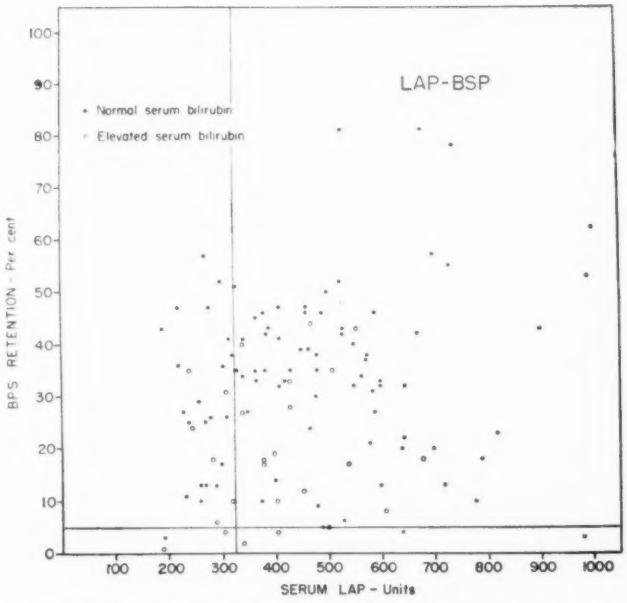

Fig. 2.—Relationship between serum LAP activity and Bromsulphalein retention in 114 serum samples. Horizontal and vertical lines indicate upper limits of normal for the two tests.

Normal pregnancy: Serum LAP activity was increased in all 15 women studied during the third to fifth month of pregnancy. In the 5 women studied during the second month of pregnancy, the values were at the upper limit of normal, ranging between 300 and 320 units. The mean for the group as a whole was significantly greater than normal ($t = 7.0$, $p < 0.01$), but was lower than in patients with hepatocellular disease ($t = 5.20$, $p < 0.01$).

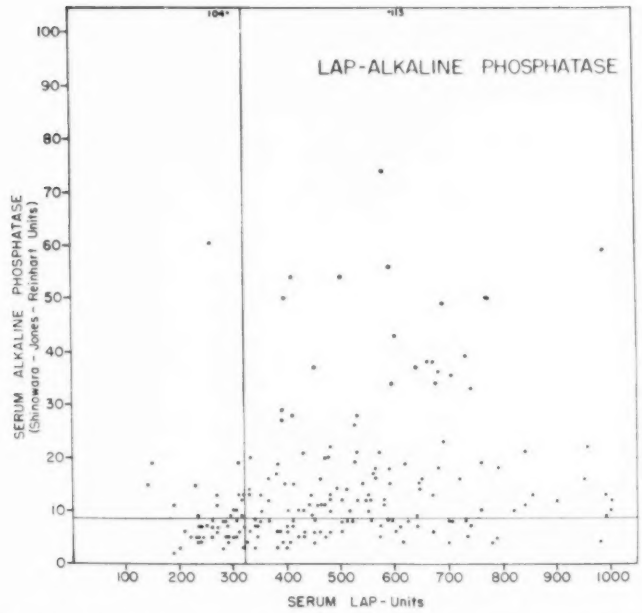

Fig. 3.—Relationship between serum LAP and alkaline phosphatase activities in 207 serum samples. Horizontal and vertical lines indicate upper limits of normal for the two tests.

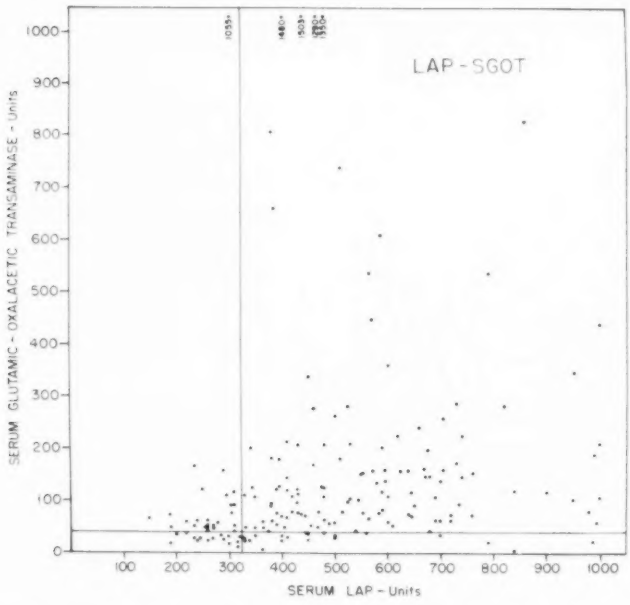

Fig. 4.—Relationship between serum LAP and glutamic oxalacetic transaminase activities in 190 serum samples. Horizontal and vertical lines indicate upper limits of normal for the two tests.

All 9 women studied at the termination of their pregnancy had increased serum LAP activity; they attained levels that were comparable to those in patients with obstructive jaundice and were significantly higher than those in patients with hepatocellular disease ($t = 2.56$, $p < 0.02$).

Acute myocardial infarction: Although serum glutamic oxalacetic transaminase activity was significantly increased in each of the 14 patients studied, only one had a raised level of serum LAP activity.

Comparison of Serum LAP Activity With Other Tests of Hepatobiliary Function.—The results of parallel determinations of serum LAP activity and other tests of hepatobiliary function are summarized in Tables III and IV and shown graphically in Figs. 1 through 5. The number of tests carried out on each sample varied, which accounts for the difference in the number of paired results reported for each combination of tests. All values of serum LAP activity for which one or more other simultaneous test determinations were available are reported; hence, the data include the results of serial determinations on individual patients and include some values obtained after recovery.

Statistically, serum LAP activity showed a significant correlation with the *serum bilirubin level* ($r = +0.205$, $p < 0.01$, $n = 210$), but, as is evident from Fig. 1, the relationship was not a close one. Indeed, there were a number of high LAP values in sera with normal bilirubin levels.

The correlation between serum LAP activity and *Bromsulphalein retention* also was statistically significant ($r = +0.190$, $p < 0.05$, $n = 114$), but again, as can be seen in Fig. 2, the relationship was not close. Moreover, the presence of jaundice appeared to be a complicating factor, since the correlation between serum LAP and Bromsulphalein retention was not statistically significant when the comparison was restricted to serum samples with a normal bilirubin content ($r = +0.90$, $p > 0.05$, $n = 26$).

The highest degree of correlation was found between the activities of serum LAP and *alkaline phosphatase* ($r = +0.398$, $p < 0.01$, $n = 207$), but even here the relationship was not close (Fig. 3).

No significant correlation could be demonstrated between the serum levels of LAP and *glutamic oxalacetic transaminase* (Fig. 4; $r = +0.129$, $p > 0.05$, $n = 190$).

The data in Table III give some indication of the relative sensitivity of the serum LAP determination in detecting the presence of hepatobiliary dysfunction. It will be observed that the results were in agreement with those of the four other tests investigated in from 63 to 77 per cent of the serum samples, in both the normal and abnormal range, and that the serum LAP determination was more sensitive than the other tests in some instances, and less so in others. Fig. 5 illustrates the same trend in each of the major disease categories investigated, viz., hepatocellular disease, obstructive jaundice caused by stone or cancer, and including the cholangitis associated with cholecystitis and pancreatitis, and hepatic metastases of both pancreatic and nonpancreatic origin.

To evaluate the statistical significance of the differences in sensitivity demonstrated in Fig. 5, the chi square method was employed, with the elimination from

TABLE III. COMPARISON OF SERUM LAP ACTIVITY WITH OTHER TESTS OF LIVER FUNCTION IN PATIENTS WITH HEPATOBILIARY DISEASE

TEST	NO. OF TESTS	SERUM LAP ACTIVITY: BOTH NORMAL (%)	BOTH ABNORMAL (%)	LAP ABNORMAL, OTHER NORMAL (%)	OTHER ABNORMAL, LAP NORMAL (%)
Serum bilirubin	210	5	67	12	16
Bromsulphalein retention	114	4	67	7	22
Serum alkaline phosphatase	207	15	48	28	8
Serum glutamicoxalacetic transaminase	190	8	69	10	13

TABLE IV. RELATIVE SENSITIVITY OF SERUM LAP ACTIVITY AND OTHER TESTS AS INDICES OF ABNORMAL HEPATOBILIARY FUNCTION IN PATIENTS WITH PROVED ACTIVE HEPATOBILIARY DISEASE

PAIRED TESTS	NO. OF TESTS	LAP ABNORMAL (%)	OTHER TEST ABNORMAL (%)	STATISTICALLY SIGNIFICANT DIFFERENCES
Serum LAP and bilirubin				
Hepatocellular disease	137	84	87	
Obstructive jaundice and cholangitis	35	71	91	$chi^2 = 4.63$, $p < 0.05$
Hepatic metastases	27	93	85	
Serum LAP and Bromsulphalein				
Hepatocellular disease	89	76	93	$chi^2 = 10.82$, $p < .01$
Obstructive jaundice and cholangitis	18	83	89	
Hepatic metastases	3	67	100	
Serum LAP and alkaline phosphotase				
Hepatocellular disease	117	93	60	$chi^2 = 36.13$, $p < .01$
Obstructive jaundice and cholangitis	33	79	70	
Hepatic metastases	25	92	92	
Serum LAP and serum glutamic oxalacetic transaminase				
Hepatocellular disease	115	86	86	
Obstructive jaundice and cholangitis	33	85	91	
Hepatic metastases	26	88	100	

consideration of paired tests which were both normal, assuming that recovery had occurred by the time they were carried out. The results are summarized in Table IV. It can be seen that the serum LAP level was a less sensitive index of obstructive jaundice and cholangitis than the *serum bilirubin* concentration, but that the two tests were equally sensitive in detecting hepatocellular disease and hepatic metastases.

Bromsulphalein retention appeared to be a more sensitive test of hepatocellular disease than was the serum LAP level. In the case of obstructive jaundice, serum LAP was as sensitive an index of disease as was Bromsulphalein retention. Unfortunately, the number of patients with hepatic metastases in whom paired tests were carried out was too small to permit a valid comparison. Accordingly, the incidence of abnormal LAP values in the entire original group of 30 patients with hepatic metastases was compared with that of abnormal

Bromsulphalein values in a comparable group of 30 nonjaundiced patients with histologically confirmed hepatic metastases in whom no serum LAP values were available. Abnormal serum LAP activity and Bromsulphalein retention were present in 28 and 27 of the 30 patients, respectively, a difference that had no statistical significance.

Serum LAP activity was more sensitive than the serum *alkaline phosphatase* level as an index of hepatocellular injury. However, in obstructive jaundice and malignant metastasis of the liver the two tests were of equal sensitivity.

In comparing the incidence of abnormal serum LAP and serum *glutamic oxalacetic transaminase* values, no significant differences were encountered in any of the disease groups studied.

Although the evidence cited indicates that the serum LAP level was about as good an index of hepatobiliary disease as any of the other tests studied, an analysis of 95 patients with well-documented hepatobiliary disease investigated by the conventional battery of tests used in such cases (serum bilirubin, alkaline

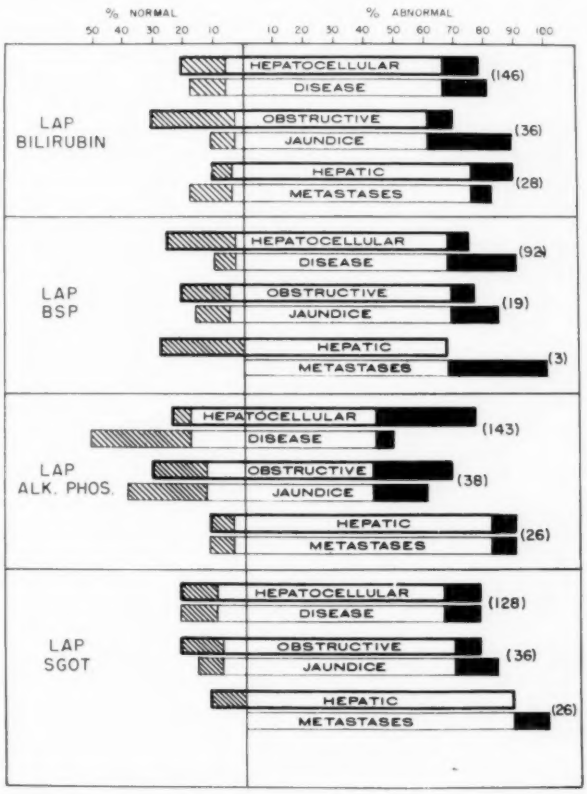

Fig. 5.—Comparison of simultaneous determinations of serum LAP activity and other tests of hepatobiliary function in various disease groups. In each disease group the upper bar indicates the serum LAP results. The solid bars represent *abnormal* values for any given test that were not in agreement with those obtained in the parallel test being compared. The cross-hatched bars represent *normal* values for any given test that were not in agreement with those obtained in the parallel test being compared. The open bars represent both normal and abnormal values that were in agreement in any pair of tests.

phosphatase, glutamic oxalacetic transaminase, Bromsulphalein retention, cephalin-cholesterol flocculation, and thymol turbidity) revealed that not in a single instance was an elevated serum LAP level the only abnormality, and, hence, of value in establishing the presence of disease. Moreover, as might be expected from the data presented in Table I, the serum LAP level proved to be of no help in differentiating between the various hepatobiliary disorders encountered in the group of 121 cases investigated.

COMMENTS

In evaluating any test of hepatobiliary function due consideration must be given to its *sensitivity* in detecting disease, its *selectivity* in differentiating between the various pathologic states that occur in the liver and biliary tree, and its *specificity* in distinguishing between hepatobiliary and other disorders.

With few exceptions the serum leucine aminopeptidase (LAP) test compared favorably in *sensitivity* with others investigated. It was less sensitive than the serum bilirubin level in obstructive jaundice, and less sensitive than Bromsulphalein retention in hepatocellular disease, but was the equal of these tests in other types of liver disease. In all categories of disease studied it equalled the sensitivity of the serum glutamic oxalacetic transaminase determination. Only in the case of serum alkaline phosphatase was it more sensitive, and then only in hepatocellular disease. In patients studied serially, serum LAP activity appeared to reflect changes in hepatic and biliary function about as accurately as did any of the other tests studied.

The great weakness of the serum LAP determination was its lack of *selectivity*. The mean for the peak values in obstructive jaundice was significantly higher than that in hepatocellular disease, but the overlap between the two groups was so great that the test proved to be of no value in distinguishing between them. Although the serum alkaline phosphatase was less sensitive than serum LAP activity as an index of hepatocellular disease, it was vastly superior in the differentiation of hepatocellular disease from obstructive jaundice, granulomatous disease of the liver, or intrahepatic tumors, values in excess of 15 units being more common in the latter groups. Similarly, serum LAP and glutamic oxalacetic transaminase activities were equally sensitive in detecting hepatocellular disease, but only the latter test was of any value in distinguishing between acute hepatitis and cirrhosis, obstructive jaundice, and infiltrative diseases of liver, values in excess of 300 serum glutamic oxalacetic transaminase units being limited almost exclusively to acute hepatitis.

The *specificity* of the serum LAP test in discriminating between hepatobiliary and other diseases would appear to be its chief virtue. On the basis of experience to date, elevated levels of serum LAP activity have been limited to hepatobiliary disease and normal pregnancy. In this respect serum LAP has proved superior to Bromsulphalein retention, serum alkaline phosphatase, serum glutamic oxalacetic transaminase, cephalin-cholesterol flocculation, and thymol turbidity, tests which are affected to a variable degree by a wide variety of extrahepatic disorders.

The suggestion of Rutenberg and associates[2] that serum LAP determinations may be of value in the diagnosis of pancreatic carcinoma was not borne out in the present study. Although all 9 of our patients with carcinoma of the pancreas had increased serum LAP activity, the levels attained were no higher than in patients with obstructive jaundice caused by common duct stone. That the increase in LAP activity was due to extrahepatic biliary obstruction in uncomplicated cases, and to a combination of obstruction and hepatic infiltration in those with hepatic metastases, is indicated by the fact that relief of biliary obstruction resulted in a prompt fall in serum LAP activity to a normal level in uncomplicated cases, but not in those with hepatic metastases. This is further supported by the observation that patients with hepatic metastases of nonpancreatic origin and without jaundice also had increased levels of serum LAP activity. It is highly probable, therefore, that the high levels reported by Rutenberg and associates[2] in patients with carcinoma of the pancreas uncomplicated by jaundice were related to the presence of hepatic metastases.

The mechanism responsible for the rise in serum LAP activity in hepatobiliary disease is not known. Fleisher and collaborators[1] have found that the leucine aminopeptidase in serum from patients with hepatitis differs from that in normal serum, and more closely resembles the enzyme found in normal human liver tissue. Accordingly, they have suggested that the liver is the source of the serum LAP in hepatobiliary disease, and that the enzyme in normal serum may be derived not only from the liver but also from other tissues. The fact that the highest levels of serum LAP activity in their study were observed in patients with acute hepatitis pointed to injured hepatic parenchymal cells as the source of the enzyme. However, both in Rutenberg's and our own studies, the levels were higher in patients with obstructive jaundice than in patients with hepatocellular disease, which suggested that the origin of the enzyme was in the biliary tree rather than in the parenchymal cells. Consistent with this possibility was the significant correlation between serum LAP and alkaline phosphatase activity and the absence of such correlation between serum LAP and glutamic oxalacetic transaminase activity observed in the present study, and the histochemical localization of the enzyme in the bile ducts of patients with high serum levels reported by Rutenberg and associates.[2]

Search for a universal test of hepatobiliary function, useful in the detection of disease, in the differentiation of the various disorders that affect the liver and biliary tree, and in measuring fluctuations in their functional status has proved to be a fruitless pursuit. As a consequence, it has become common practice to employ a combination of tests selected for these special purposes. Although many tests have proved their worth and thus gained wide acceptance, it is well known that even in combination they often fall short of the objectives set for them, particularly in distinguishing between the various disorders that affect the hepatobiliary system. This accounts for the inordinate interest in new tests which continue to appear at an ever increasing rate. Although efforts to improve and amplify such diagnostic methods are to be encouraged, there is the danger that their premature adoption may add to the financial burden of

the patient, overwhelm laboratory facilities, and confuse the clinician without adding any useful information. The determination of serum LAP activity is a case in point. On the basis of its sensitivity and relative specificity, it would appear to merit acceptance as a diagnostic test. However, it has no value in differentiating between the various disorders that affect the hepatobiliary system, and adds no useful information to that already provided by the group of tests now in common use. Accordingly, there would appear to be little justification for adding the determination of serum leucine aminopeptidase activity to the already long list of diagnostic tests used in the investigation of hepatobiliary disease.

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COMPARISON OF EFFECT OF CEREBROSPINAL FLUID AND OF BLOOD SERUM ON TISSUE CULTURES

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Blood sera and cerebrospinal fluids from schizophrenic and preoperative surgical patients were tested for their effects on strain L cells in cultures. It was found that in spite of the fact that blood serum would kill the cells, the cerebrospinal fluid from the same patient remained nontoxic even when used in 100 per cent concentration. These observations, combined with some previous ones, suggest that the toxic substances in the blood serum probably are not present in the cerebrospinal fluid.

PREVIOUSLY I have reported that by using tissue culture methods, toxic substances are demonstrable in some human blood sera.[1-4] Neither the origin nor the significance of these substances in vivo is known. For that reason it seemed worth while to investigate whether or not these substances are present in body fluids other than blood serum. This report describes the investigations of cerebrospinal fluids of patients who have substances in their blood serum which are toxic to strain L cells.

METHODS

Source and Preparation of Cerebrospinal Fluids and of Blood Sera.—Previously we found that in a very high percentage of schizophrenic patients and of preoperative patients the blood serum is toxic to strain L cells.[2] For our present study we obtained cerebrospinal fluids and blood from mental patients in the Saskatchewan Hospital, North Battleford, and in the Psychiatric Ward of the University Hospital, Saskatoon, and from surgical patients in the Surgical Ward of the University Hospital, Saskatoon. The samples were always taken from the mental patients before they had breakfast and from surgical patients before anesthetics were given. All syringes, needles, and test tubes were cleaned by the method devised by Hanks for cleaning glassware for tissue culture purposes[5] and sterilized. The test tubes were stoppered with nontoxic rubber stoppers.[6] Special care was taken to avoid taking samples from mental patients who had complicating conditions, such as infection or menstruation, or who had recently received medications. In the case of surgical patients such precautions were not taken.

The cerebrospinal fluid and blood samples, which were shipped immediately to Saskatoon from the Saskatchewan Hospital in North Battleford, were kept chilled, but not frozen, by packing the test tubes in large vacuum bottles which contained ice. On arrival, usually not more than 24 hours after shipping, the blood was centrifuged, and the serum was removed and stored at −20° C. until used. Cerebrospinal fluid was stored at −20° C. without previous centrifugation. Serum and cerebrospinal fluid obtained locally were also stored at −20° C.

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Cell Cultures.—In these studies the so-called "strain L" cell cultures were used. Strain L had been established in 1940 by Earle and his associates at the National Cancer Institute in Bethesda.[7, 8] They explanted and cultured subcutaneous tissue of a normal C_3H male mouse, from which cultures a number of sublines were obtained and treated for various periods of time with 20-methylcholanthrene. In this way "fibroblastlike" cells were obtained which, on injection into normal C_3H mice, produced sarcomas.[9] In 1948 Sanford and co-workers[8] isolated a single cell from one of the sarcomas, producing a subline which they designated "NCTC-clone strain L."[10] Stock cultures of NCTC-clone strain L cells are grown in our laboratory at 37° C. in a medium which consists of 2 parts horse serum, 3 parts synthetic medium 1066,* and 5 parts Hanks' balanced salt solution.[5] The medium is completely changed twice weekly and the cells are transplanted into new bottles every 8 to 10 days.

Procedure of Toxicity Test.—The tests reported here were made in Leighton tubes.† Into each Leighton tube 1.5×10^5 cells from the stock cultures were inoculated and the Leighton tubes were incubated for 2 or 3 days. The medium was removed and cerebrospinal fluid or blood serum was added, after dilution to 50 per cent concentration with balanced salt solution. The cultures were incubated for 3 days and the effect on the cells of the cerebrospinal fluid or of blood serum was evaluated each day. The evaluation was made as follows: zero (0), when no toxic effect could be detected, 1 plus (+), when only a few rounded-up degenerating cells were seen, 2 plus (++), when the degenerating or dead cells had increased in number but healthy cells still predominated in most microscopic fields, 3 plus (+++), when there were more degenerating or dead cells than healthy cells, and 4 plus (++++), when all but a few scattered cells were dead.[1] The final serum toxicity was calculated by averaging the results of evaluation of the cultures after 24, 48, and 72 hours of incubation. At least two replicate cultures were used each time.

RESULTS

Cerebrospinal fluids from 19 schizophrenic patients and from 40 preoperative patients were tested for the effect on strain L cells. Table I shows that from 19 cerebrospinal fluids obtained from schizophrenic patients only one showed a toxicity of 2 plus (++), and from 40 cerebrospinal fluids from preoperative patients, one had a toxicity of 2 plus (++) and four of 1 plus (+).

In the second series of tests, mental patients and preoperative patients who had very toxic serum (4 plus) were selected and their blood sera as well as cerebrospinal fluids in 50 per cent concentration in balanced salt solution were tested on strain L cells. Table II shows that the cerebrospinal fluid from these patients was completely nontoxic to strain L cells.

TABLE I. EFFECT OF CEREBROSPINAL FLUID ON STRAIN L CELLS

CEREBROSPINAL FLUID FROM	NO. OF CASES	NO. OF PATIENTS HAVING DEGREE OF TOXICITY OF				
		0	+	++	+++	++++
Schizophrenic patients	19	18	—	1	—	—
Preoperative patients	40	35	4	1	—	—

*Synthetic medium CMRL-1066 is a modification of medium 858.[25] The modification is that from medium 858 the six highly purified coenzymes, ferric nitrate, and fat-soluble vitamins (A, D, E, and K) have been omitted, and that five B vitamins and Armour's liver (porcine) coenzyme have been added. The medium CMRL-1066 was obtained from the Connaught Medical Research Laboratories, University of Toronto, Canada.

†Leighton tubes are test tubes with one area of the wall flattened. Cells are cultured on the flattened area.

TABLE II. EFFECT OF CEREBROSPINAL FLUID AND BLOOD SERUM FROM THE SAME PATIENTS ON STRAIN L CELLS

NO.	PATIENT	DIAGNOSIS	TOXICITY OF	
			SERUM	CEREBROSPINAL FLUID
1	JC	Schizophrenia	++++	0
2	HB	Schizophrenia	++++	0
3	EP	Schizophrenia	+++	0
4	WR	Schizophrenia	++++	0
5	BE	Schizophrenia	++++	0
6	MeN	Neurosis	+++	0
7	WB	Prostatic hypertrophy	++++	0
8	KE	Prostatic hypertrophy	++++	0
9	LH	Fibroids	++++	0
10	CS	Hernia	++++	0
11	CC	Hernia	++++	0
12	TD	Varicose veins	++++	0

Since it appeared possible that the concentration of the toxic substances in the cerebrospinal fluid might be low, cells were also grown in a medium which consisted entirely of cerebrospinal fluid. No toxic effect was observed, even when the cerebrospinal fluid was taken from patients whose blood serum was highly toxic. (Fig. 1).

DISCUSSION

Other investigators, using various biologic test methods, have investigated the presence of toxic substances in the cerebrospinal fluid of schizophrenic patients. Gamper and co-workers[11] found that injection of cerebrospinal fluid from schizophrenic patients and from patients with organic brain diseases, into the anterior chamber of the eyes of rabbits, caused inflammatory changes, whereas no change or very little was found when cerebrospinal fluid from healthy persons was injected. According to Gamper and Kral[12] the majority of white mice injected subcutaneously with cerebrospinal fluid from schizophrenic patients and from patients with organic diseases of the central nervous system died after 3 to 4 days. Cerebrospinal fluid from healthy persons and from patients with nonorganic diseases was less toxic. Lethal cerebrospinal fluid was found most frequently among catatonic patients, then among hebephrenic patients, and least frequently among paranoiac patients. The cerebrospinal fluid from patients with acute schizophrenia was more toxic than that from chronic schizophrenic patients. In the patients with chronic cases, however, the actual duration of the disease seemed not to influence the toxicity of the cerebrospinal fluid. The injection of cerebrospinal fluid from schizophrenic patients induced cramps in the animals, and on postmortem examination the authors found scattered foci of liquefaction necrosis throughout the brain as well as some degenerative changes in the liver.

Reiter[13] tried to reproduce the findings of Gamper and Kral by injecting, subcutaneously, cerebrospinal fluid from schizophrenic patients into white mice. He injected 138 cerebrospinal fluids into several hundred mice, and saline solution into mice of a control group. His results did not support the observations of Gamper and Kral, and he suggested that the difference in results

might be due to a difference in the sensitivity of the mice used, or to the use of nonsterile cerebrospinal fluid by Gamper and Kral. Not being entirely convinced by the results obtained by the subcutaneous injections of cerebrospinal fluid in mice, Reiter asked Fischer to test cerebrospinal fluid from schizophrenic and nonschizophrenic patients on cells grown in tissue cultures. In these tests Fischer used human osteoblast cultures and evaluated growth by measuring the area of outgrowth with a planimeter. Fischer tested 20 cerebrospinal fluids from schizophrenic patients and 8 cerebrospinal fluids from nonschizophrenic mental patients by comparing the growth of cultures in the cerebrospinal fluids to the growth of cultures in balanced salt solution. The results showed that between the cerebrospinal fluids of schizophrenic patients and those of nonschizophrenic mental patients, there was no significant difference as to their influence on the growth of human osteoblast cultures. When the growth in cerebrospinal fluid was compared to the growth in balanced salt solution it was found that cerebrospinal fluid was not toxic and that in many instances the osteoblasts in cerebrospinal fluid formed even larger outgrowth areas than those in balanced salt solution. From these experiments and from his injections of cerebrospinal fluid into young mice, Reiter concluded that there are no toxic substances in the cerebrospinal fluid of schizophrenic patients.

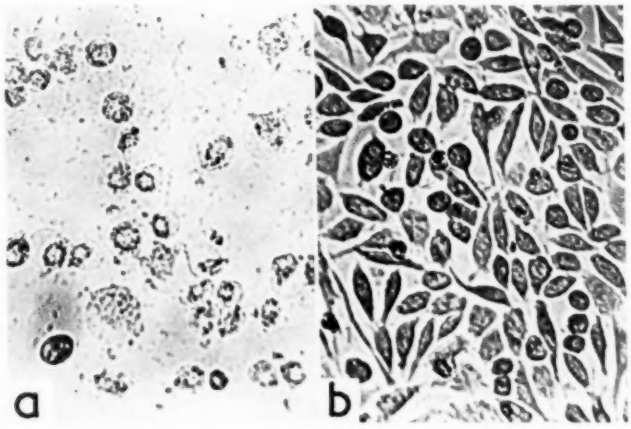

Fig. 1.—Effect on strain L cells of blood serum and cerebrospinal fluid obtained at the same time from the same patient. *a*, Effect of 25 per cent blood serum in balanced salt solution. *b*, Effect of 100 per cent cerebrospinal fluid. (Magnification ×400; reduced 2/3.)

Similarly, Scheid[14] was unable to duplicate the work of Gamper and Kral and suggested that their mice colonies may have had an epidemic infection which influenced the results. Sogliani[15] tested cerebrospinal fluids from patients with acute and chronic schizophrenia by intraperitoneal injections into rabbits and mice and found no difference in the effect of the fluids.

Le Grand and Année[16, 17] reported that rats showed catatonic symptoms when given subcutaneous injections of the residue obtained by evaporating, at not more than 37° C., cerebrospinal fluid obtained from schizophrenic patients.

This effect lasted from a few hours to more than 48 hours. Some animals died after the injection. The cerebrospinal fluid from nonschizophrenic patients, prepared in the same way, produced no catatonic symptoms when injected into rats. These authors also found that the catatonogenic substance in cerebrospinal fluid from schizophrenic individuals was inactivated by heating at 45° C. for 30 minutes. It was destroyed by cold, its activity slowly disappeared at room temperature, and it was nondialyzable. Recently Shapiro[18] reinvestigated this work. He lyophilized the cerebrospinal fluid and measured the effect of the injected concentrated cerebrospinal fluid on rats by means of "rat-activity wheels." He was unable to detect the presence of a catatonogenic substance in cerebrospinal fluid of schizophrenic patients. He did notice that after the injection the motor activity of the rats decreased, but he showed that the decrease was due to the hypertonicity of the injected solution rather than to a catatonogenic substance.

Macht[19] found that the growth of the roots of *Lupinus albus* (white lupin) in 2 per cent solution of cerebrospinal fluid from schizophrenic patients was markedly inhibited, but that there was no or only slight inhibition of growth when such plants were grown in cerebrospinal fluid from healthy persons. Cerebrospinal fluid from patients with general paresis was as toxic as cerebrospinal fluid from schizophrenic patients, whereas cerebrospinal fluid from patients with tabes dorsalis or neurosyphilis was only slightly toxic and did not differ significantly from cerebrospinal fluid from healthy persons.[19, 20] Katzenstein-Sutro and Staub[21] found that cerebrospinal fluid from schizophrenic and neurotic patients inhibited the growth of *Lipidium sativum* (garden cress) less than did cerebrospinal fluid from epileptic individuals and from patients with organic brain diseases. Since Katzenstein-Sutro and Staub did not use the same plant as test object as Macht did, it is difficult to compare their findings.

Recently Mastrogiovanni and Scarlato[22] reopened the problem of the toxicity of cerebrospinal fluid in schizophrenic patients and reinvestigated the problem with techniques used in virology. They injected cerebrospinal fluid into the amniotic sac of 11-day-old chick embryos. When cerebrospinal fluid from schizophrenic patients was injected (21 samples), 70 per cent of the embryos died, whereas with cerebrospinal fluid from healthy persons, only 8 to 12 per cent of the embryos died. When the fluid removed from the allantois of the injected embryos was passed through chick embryos seven times, the mortality of embryos increased up to 88 per cent. Passage of fluid from the allantois of the embryos injected with cerebrospinal fluid of healthy persons did not increase the number of deaths. Examination of dead embryos showed hemorrhages in the peritoneum and in the walls of the stomach and heart, and subpial hemorrhages in the brain. Examining by electron microscopy the allantoic fluid of embryos injected with cerebrospinal fluid of schizophrenic patients, Mastrogiovanni and Scarlato found small spherical particles, singly or in groups. There were no such particles seen in the fluid taken from the allantois of the control embryos. From these experiments they concluded that viruslike bodies are present in the cerebrospinal fluid of schizophrenic patients. Subsequently, Scarlato and Mastrogiovanni[23]

injected guinea pigs with fluid taken from the allantois of embryos previously injected with cerebrospinal fluid from schizophrenic patients. None of the controls died, but 75 per cent of the injected animals did. Mice died in 40 per cent of cases when the same fluids from the allantois were injected into the vitreous body of the eye. By means of the electron microscope these workers also observed viruslike particles in the homogenate of the vitreous body of the eyes of the injected white mice. In a more recent investigation, however, Mastrogiovanni[24] tested the cerebrospinal fluids and washings from the rectum and pharynx of schizophrenic patients on monkey kidney cell cultures. Although the fluids had a pathogenic effect on the cells, which was intensified after passage through chick embryos, he was unable to identify the pathogenic effect with the cytopathogenic effect of any known virus.

My finding, that cerebrospinal fluid of schizophrenic patients is not toxic to strain L cells, supports the findings of Fischer,[13] Reiter,[13] Scheid,[14] Shapiro,[18] and Katzenstein-Sutro and Staub,[21] but not those of Gamper and associates,[11, 12] Le Grand and Année,[16, 17] Macht,[19, 20] and Mastrogiovanni and Scarlato.[22-24] However, it is difficult to compare my results to those of other investigators because of the variety of test objects used.

The observation, that substances causing death of strain L cells in cultures occur in some blood serum but not in cerebrospinal fluid or at least not in demonstrable quantities, suggests that these substances do not penetrate the blood–cerebrospinal fluid barrier. This does not, however, exclude the possibility that they may reach the brain proper in places where there is no cerebrospinal fluid between the blood vessel and the nerve tissue or where there is little perivascular cerebrospinal fluid. In the latter case it is possible that the substances entering the perivascular cerebrospinal fluid would enter the brain before diffusing through all the cerebrospinal fluid, thus being indetectable in the cerebrospinal fluid obtained by lumbar puncture. It seems very unlikely, however, that the toxic substances could penetrate the blood-brain barrier. One reason for the unlikeliness is the earlier finding that the toxic substances in the serum are nondialyzable, which indicates that they are either large molecules or attached to large molecules.[3] It is generally believed that large molecules do not penetrate the undamaged blood-brain barrier.

The actual nature and significance of the toxic substances in the blood serum still remain to be found.

SUMMARY

Cerebrospinal fluids from schizophrenic and preoperative patients were not toxic to strain L cells. Cerebrospinal fluids from patients with very toxic blood serum was also nontoxic to strain L cells in concentrations of either 50 or 100 per cent.

I am indebted to Dr. A. B. Dobkin, Department of Anesthesia, University Hospital, Saskatoon, for obtaining cerebrospinal fluids and blood from preoperative patients, and to the staffs of the Saskatchewan Hospital in North Battleford and the Department of Psychiatry, University Hospital, Saskatoon, for providing the cerebrospinal fluids and blood from mental patients.

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USE OF RADIOIODINATED HIPPURAN FOR INDIVIDUAL KIDNEY FUNCTION TESTS

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External radioactivity counting over each kidney area is the most rapid, simple, and atraumatic method available for simultaneous comparison of kidney function. However, radioactive substances presently used for this purpose have major disadvantages: iodopyracet (Diodrast) is partly removed from the blood by the liver, which necessitates complicated techniques for viewing the kidneys in order to exclude liver radioactivity; such materials as sodium diprotrizoate (Miokon), diatrizoate methylglucamine (Renografin), and diatrizoate sodium (Hypaque sodium) are removed from the blood slowly, thus prolonging test time and obscuring differences in kidney function.

Radioiodinated o-iodohippurate (Hippuran) has all the advantages of these materials with none of the disadvantages. It is rapidly and selectively removed from the blood by the kidneys. Since the liver does not take up Hippuran, wide-angle "blind" collimation may be used, simplifying the test procedure. Rapid extraction of Hippuran from the blood by the kidneys reduces the test time and amplifies differences in kidney function.

EXTERNAL radioactivity counting over the kidneys is the simplest and least traumatic method for comparing individual kidney function. However, labeled substances presently used for this purpose have major disadvantages. I^{131}-labeled Diodrast, the first such substance used, is in part removed from the blood by the liver, thus requiring critical probe manipulation to view the kidneys without including liver radioactivity.[1, 2] Diodrast has currently been replaced by other labeled materials such as sodium acetrizoate (Urokon sodium), Miokon, Hypaque sodium, and Renografin since they are not appreciably taken up by the liver.[3] This allows probes to be placed at right angles to the back with relatively wide collimation and without recourse to x-ray localization of the kidneys. Although these materials represent an improvement over Diodrast, they have the disadvantage that they are removed from the blood much more slowly than is Diodrast, which prolongs test time and dulls the detection of kidney function differences.

Theoretic considerations led us to predict that *o*-iodohippurate sodium (Hippuran) would have ideal characteristics for comparing individual kidney function, i.e., rapid removal from the blood by the kidneys only. Further, since nonradioactive Hippuran contains an iodine atom, I^{131} can be substituted for it. One of us (M. T.) has prepared the labeled Hippuran in this laboratory, and the present studies[4] were carried out to determine its clinical usefulness.

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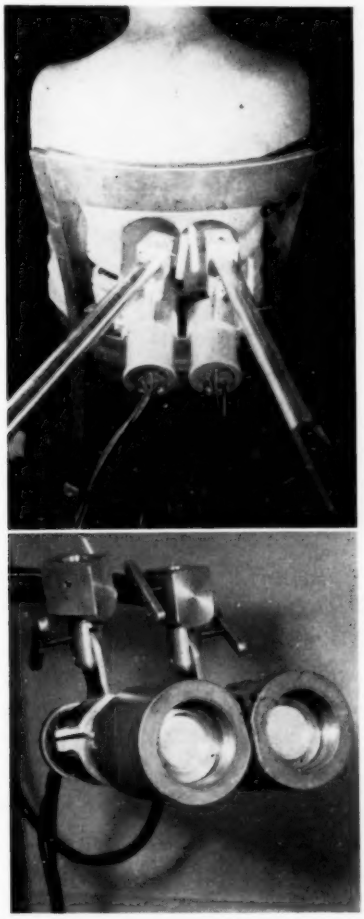
METHOD

Sodium radioiodohippurate was prepared by an exchange reaction between sodium radioiodide and sodium *o*-iodohippurate at pH 6.0 and 100° C. The free radioiodohippuric acid was isolated from the sodium salt, in the presence of small amounts of carrier iodide, to a constant specific activity of 50 μc per milligram.[7] Two matched scintillation probes were used, each attached to a rate meter and a rectilinear recorder. The crystals used were 1 by 1 inch, recessed 1½ inches within ⅝ inch lead shields, with 2½ inch apertures. Before each test a 1 μc I^{131} source was centrally placed before each probe to assure that the recorded responses were identical. The rate meter time constant was 15 seconds and the entire paper width represented 1,200 counts per second. The subject was placed in a comfortable sitting position, after which the probes were positioned parallel to each other at right angles to the back, centered over the kidneys (Fig. 1). This position was approximated by measuring four fingerbreadths above the left iliac crest for the left probe, and 2 cm. lower than this for the right. The dose was calculated on the basis of 0.4 μc per kilogram body weight. This was contained in 0.5 to 5.0 mg. of radioactive Hippuran, 0.1 to 0.3 mg. of Miokon, and 0.25 to 1.0 mg. of Diodrast. The test substance was injected intravenously and the recorder was started.



A.

B.

Fig. 1.—*A*, Position of probes over the kidney areas. *B*, Close-up of probes.

RESULTS

1. *Kidney Curves in Normal Subjects: Hippuran Versus Miokon.*—After an initial spike the curves obtained externally over the kidney areas with Miokon rose slowly and reached a peak at 6 to 8 minutes, followed by a decline. The Hippuran curves rose more rapidly, reached a peak at 2 to 5 minutes, and then declined rapidly (Fig. 2).

2. *Hydration and Dehydration: Hippuran Versus Miokon.*—We have observed marked changes in the shape of kidney curves obtained with Miokon in subjects with both normal and abnormal kidney function, often of sufficient magnitude to confuse the interpretation. Figs. 3 and 4 are examples of results obtained in the same individual with hydration (1,200 ml. water in 2 hours preceding the test) and dehydration (no food or water for 16 hours prior to the test). In the hydrated state with Miokon the rising portion of the curve is relatively short, reaching a peak in 4 minutes and decreasing fairly rapidly thereafter. In the dehydrated state the rising portion of the curve is longer, reaching a peak at 7 minutes. The Hippuran curves demonstrate changes in the same direction but the differences appear less pronounced.

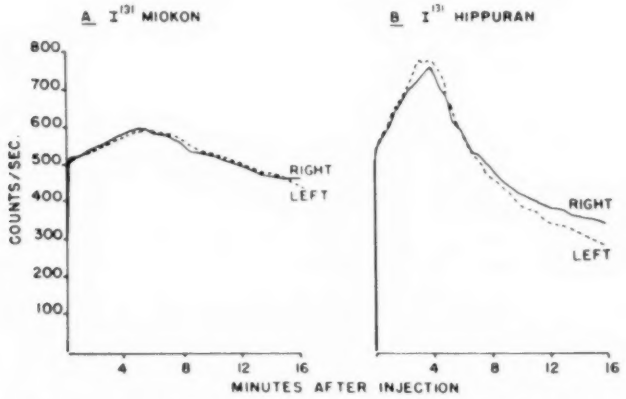


Fig. 2.—I^{131}-labeled Miokon and Hippuran in the same normal subject.

3. *The Surgically Absent Kidney.*—Two years prior to this test the patient's left kidney was removed because of an infarction which caused arterial hypertension. Subsequent blood pressures have been within normal limits and a recent serum creatinine was 1.2 mg. per cent. Despite relatively wide collimation little scattered activity from the right kidney is noted on the left (Fig. 5).

4. *Nonfunctioning Kidney: Hippuran Versus Miokon Versus Diodrast.*—I. E. is a 40-year-old normotensive man with a nonfunctioning right kidney as a result of trauma. A recent intravenous urogram showed normal visualization of the left pelvis and calyces with prompt excretion into the bladder; no visualization occurred on the right. The serum creatinine was 1.3 mg. per cent. Miokon curves (Fig. 6, *A*) indicate good radioactivity uptake on the left but essentially none on the right. A repeat study under similar hydration

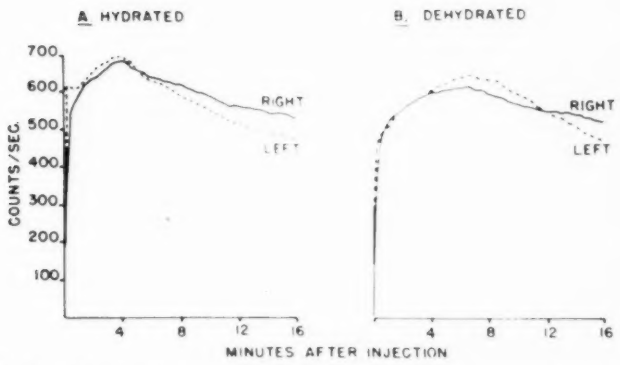


Fig. 3.—Hydration and dehydration in a normal subject with Miokon.

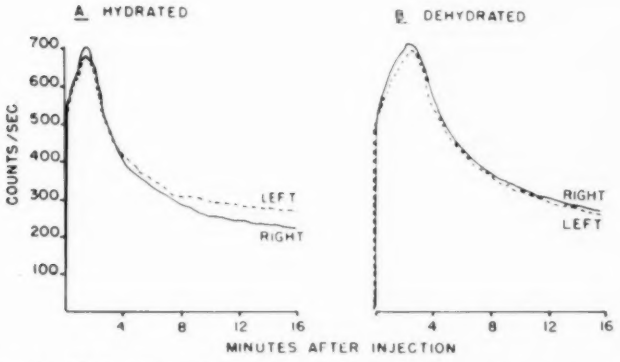


Fig. 4.—Hydration and dehydration in a normal subject with Hippuran.

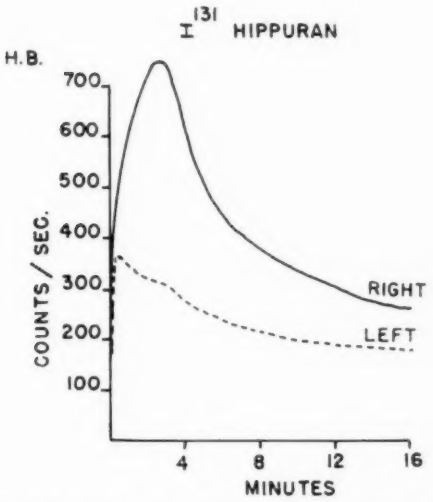


Fig. 5.—Surgically absent left kidney using Hippuran.

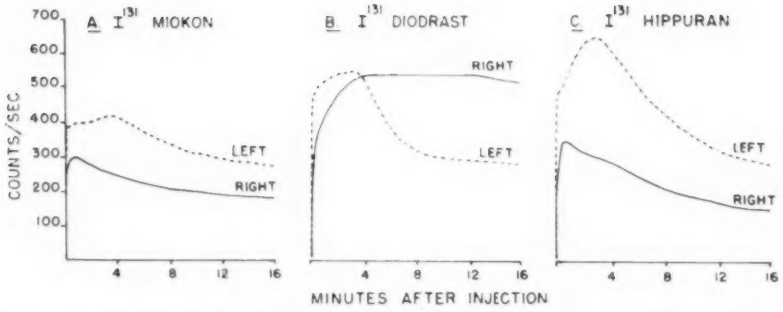


Fig. 6.—Nonfunctioning right kidney. Comparison of Miokon, Diodrast, and Hippuran.

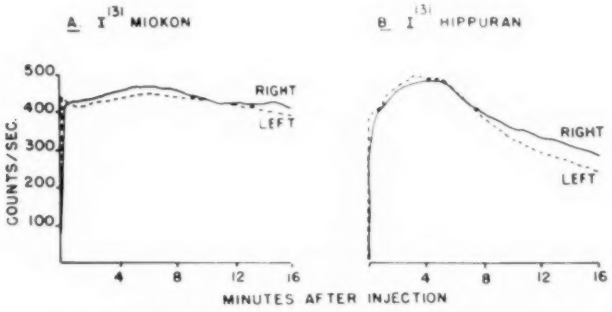


Fig. 7.—Mild hypertension. Miokon versus Hippuran.

conditions with Diodrast (Fig. 6, *B*) indicates rapid uptake on the left and uptake on the right which was not noted with Miokon. Hippuran curves (Fig. 6, *C*) demonstrate good uptake on the left and essentially none on the right. It is evident from these curves that Diodrast is taken up by the liver whereas Miokon and Hippuran are not, and that Hippuran is more rapidly extracted from the blood by the kidneys than is Miokon.

5. *Mild Hypertension: Hippuran Versus Miokon.*—A patient with mild hypertension was studied in order to compare Hippuran and Miokon (Fig. 7). His blood pressure was 170/100 mm. Hg, serum creatinine 1.6 mg. per cent, phenolsulfonphthalein excretion 22 per cent in 45 minutes. There was decreased accumulation of activity over the kidney areas with both labeled materials compared to the respective normals, but more uptake occurred with Hippuran.

6. *Severe Hypertension: Hippuran Versus Miokon.*—This patient was a 33-year-old man who entered the hospital with a blood pressure of 250/150 mm. Hg and serum creatinine of 3.8 mg. per cent. At the time of the test the blood pressure was 180/110 mm. Hg and the creatinine level was 3.2 mg. per cent. Miokon curves indicate very little radioactivity uptake by the kidneys and are suggestive of recordings obtained over the abdominal aorta (Fig. 8). The uptake with Hippuran was more marked but occurred slowly; the activity peak was reached much later than normal and was followed by a slow decline.

7. *Decompensated Unilateral Kidney Function: Hippuran Versus Miokon.*—Fig. 9 shows the results obtained in a patient with a tumor of the bladder. An intravenous urogram showed some opacity of the pelvis of the left kidney at 15 minutes but none on the right. The serum creatinine was 2.8 mg. per cent. Data obtained with Miokon does not suggest function of either kidney, although the curve obtained over the left kidney area is somewhat higher than that obtained over the right. Hippuran, however, indicates a definite uptake of activity on the left as compared to the right. A subsequent operation demonstrated a complete block of the right ureter by tumor with a patent left ureteral orifice.

8. *Blood Disappearance Rates.*—The disappearance rate of labeled Hippuran from the blood is more rapid than that of Miokon. Using the 5 minute blood radioactivity level as 100 per cent, the 15 minute level of Hippuran in a normal subject was 58 per cent, whereas in the same individual the 15 minute level of Miokon was 73 per cent. This compares well with other data obtained by external counting over the high anterior chest.

9. *Urine Excretion.*—The average 30 minute urine collection in 3 normal subjects contained 71 per cent of the injected dose of Hippuran and 34 per cent of the injected dose of Miokon.

10. *Bile Excretion and Thyroid Uptake.*—In one patient who had undergone a recent cholecystectomy, 0.04 per cent of the injected Hippuran activity

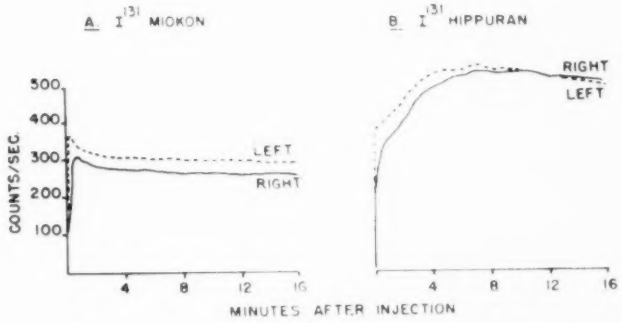
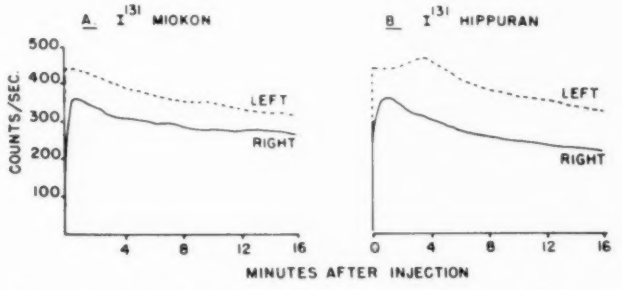


Fig. 8.—Severe hypertension. Miokon versus Hippuran.



9.—Nonfunctioning right kidney with poorly functioning left kidney. Miokon versus Hippuran.

was recovered from a 20 minute collection of free-flowing T tube bile. The thyroid uptake of I^{131} at 24 hours in 2 individuals was less than 1 per cent in each.

DISCUSSION

The need for a valid, rapid, atraumatic method for comparing individual kidney function is evident, and the use of external radioactivity counting over each kidney area has appeared to have merit. However, it has been beset by difficulties. Labeled Diodrast, because of hepatic uptake, requires complicated collimation. Labeled Urokon, Miokon, Hypaque sodium, and Renografin are cleared more slowly by the kidneys,[1] which prolongs the test time to 15 to 25 minutes and dulls observation of kidney function differences. Despite these difficulties, results have in general been promising.

I^{131}-labeled Hippuran was prepared in this laboratory on the prediction that it would have the advantages without the disadvantages of previously used substances. Nonradioactive Hippuran is known to be cleared from the blood almost completely in one passage through the kidney, and for this reason has been used to determine renal plasma flow.[5] Since chemical means for quantitating iodine are difficult, para-aminohippurate has been substituted for it in standard kidney function studies. The iodine atom in Hippuran, however, makes it relatively simple to radioiodinate by an exchange reaction and thus it appears to be preferable to para-aminohippurate for the present purpose. Its toxicity is low: 20 to 30 Gm. of the substance has been used intravenously in adults without reaction,[6] whereas the maximum weight of our material is 0.005 Gm.

Tagged Hippuran is not appreciably taken up by the liver, which allows probes to be placed at right angles to the back directly over the kidneys and greatly simplifies collimation. The most convincing evidence of lack of liver uptake is the similarity of curves obtained over kidneys which are functioning equally, in contradistinction to the curves obtained with Diodrast in which the right curve is increased over the left (see Fig. 6). Additional evidence is provided by the virtual absence of radioactivity in T tube bile.

Demonstration of equal uptake in equally functioning kidneys is dependent in part on proper tube placement. If one probe is placed directly over a kidney and the other is not, different curves will result which may be incorrectly interpreted as a difference in kidney or excretory function. Large probe apertures, providing a large area of "vision," minimize this problem without appreciable distortion from the contralateral kidney (see Fig. 5). Several methods are available for positioning the probes. The standard method is to localize the kidneys by an upright x-ray of the abdomen. This is accurate but costly and time consuming. A simpler and perhaps even more accurate method has been suggested by Frohlich and associates.[2] This involves the pre-injection of a small dose of the kidney testing material and positioning the probes by locating the maximum point of uptake on each side separately. This technique requires a separate injection and confuses external counting if simultaneous bladder and blood data are being obtained, but is perhaps

the method of choice. We have used a third method with some success: the probes are positioned by the usual blind technique, and after the test is finished the probes are individually raised and then lowered to assure that maximum response is present over the site used for the test. If it is not, a repeat test may be necessary. It should be recognized that in low-lying kidneys both positioning and curve data may be confused by bladder radioactivity.

The rapidity with which the kidneys remove labeled Hippuran from the blood has special advantages. Besides shortening the test to less than 10 minutes, the rapid extraction magnifies both the absolute function and the differences in function between the two kidneys. This is especially evident in severely diseased kidneys. Comparison between this procedure and the intravenous urogram has not been adequately tested, but on theoretic grounds as well as in a few comparisons (e.g., Fig. 9) it appears that labeled Hippuran is at least as sensitive as the standard urogram and will, therefore, be useful for screening purposes.

SUMMARY

For simultaneous comparison of kidney function by external radioactivity counting, I^{131}-labeled sodium *o*-iodohippurate (Hippuran) eliminates the major disadvantages of currently used substances. It is not appreciably removed from the blood by the liver, as is labeled Diodrast, thus allowing simplified collimation to be used. Since it is extracted from the blood by the kidneys more rapidly than such labeled substances as Urokon, Miokon, Hypaque sodium, and Renografin, testing time is reduced. In addition, studies in patients with known pathologic conditions of the urinary tract indicate that labeled Hippuran demonstrates comparative differences more sensitively and more accurately than do substances with slower blood extraction rates.

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EFFECT OF LIVER DISEASE ON DIIODOTYROSINE METABOLISM

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It would appear that liver disease has little effect upon the rate with which intravenously administered I^{131}-labeled diiodotyrosine (DIT) is metabolized, although small but significantly larger amounts of diiodotyrosine are excreted into the urine of patients with hepatic dysfunction. Since I^{131}-labeled diiodotyrosine is deiodinated very rapidly both in control subjects and in persons with liver disease, most of the I^{131} appearing in the plasma, urine, and saliva after 10 to 15 hours is in the form of iodide. Therefore, the study is reduced to one of an evaluation of the effects of hepatic dysfunction on iodide metabolism. Chromatographic separation of the I^{131}-labeled substances appearing in plasma, saliva, and urine samples enabled us to plot curves of diiodotyrosine disappearance and iodide appearance and eventual disappearance from these tissues. I^{131} iodide disappearance rate constants were calculated from these curves, and it was concluded that the I^{131}-labeled iodide which results from the deiodination of diiodotyrosine is cleared at a slower rate in persons with liver disease. This observation may be explained by a decreased rate of I^{131} iodide excretion by the kidneys.

DEIODINATION is apparently one of the first steps observed in the in vivo degradation of diiodotyrosine (DIT), although small amounts of the corresponding pyruvic and lactic acid derivatives of diiodotyrosine have also been observed in the urine of experimental animals.[1, 2] Generally, the concentration of these substances in urine does not exceed 10 per cent of the injected dose of I^{131}-labeled diiodotyrosine.[1, 2] Rapid deiodination of diiodotyrosine has also been observed in human subjects,[3, 4] and consequently, the rate and completeness with which diiodotyrosine is deiodinated might be studied and used as a measurement of diiodotyrosine catabolism in certain metabolic disorders.

Roche and coauthors[5] have postulated that the thyroid gland itself may be the most active tissue involved in the catabolism of diiodotyrosine. However, Tong and associates[2] found that thyroidectomy had little if any effect on the deiodination of diiodotyrosine in the intact rat and Albert and Keating[3] observed that diiodotyrosine deiodination takes place just as rapidly and completely in treated subjects with myxedema as in normal control subjects. More recently, Stanbury and co-workers[6, 7] have shown that patients with hypothyroidism and familial goiter deiodinate diiodotyrosine less completely than do normal subjects. Therefore, the role of the thyroid in diiodotyrosine deiodination is still in doubt.

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Other organs such as the liver and kidney may deiodinate diiodotyrosine. Relatively large amounts of diiodotyrosine deiodinase have been demonstrated in these organs in in vitro tissue incubation experiments.[5, 8-10] Since studies in this laboratory have shown that diiodotyrosine is deiodinated just as completely in the dog which has undergone nephrectomy as in the intact animal, it would appear that the liver might be more important than the kidney in diiodotyrosine deiodination. Liver disease might therefore be expected to affect the catabolism of this amino acid. To test this hypothesis, a kinetic model was devised for following whole body diiodotyrosine deiodination,[11] and this model has been employed in the present study of the relationship between liver disease and diiodotyrosine deiodination.

PATIENTS AND METHODS

One hundred microcurie carrier-free doses of I^{131}-labeled diiodotyrosine* were administered intravenously to two groups of patients. The control group was composed of eight individuals without clinical or laboratory evidence of thyroid, liver, or kidney disease who were hospitalized for minor illnesses. The experimental group consisted of nine patients with hepatomegaly and associated hepatic dysfunction as evidenced by the following liver function values: Bromosulphalein retention, 16 to 72 per cent; cephalin flocculation, 1 plus to 4 plus; and bilirubin, 0.3 to 5.1 mg. per cent. Total plasma protein concentration ranged between 6 and 8 Gm. per cent with inversion of the albumin to globulin ratio in three subjects. The liver was enlarged in each case, extending from 3 to 12 cm. below the costal margin in the midclavicular line. Ascites was present in two patients. In the majority of cases, the clinical features and laboratory data were consistent with the diagnosis of Laennec's cirrhosis.

Heparinized blood samples and 3 minute saliva specimens (obtained under the stimulation of gum chewing) were collected 15 minutes after injection of the I^{131}-labeled diiodotyrosine and at frequent intervals thereafter. Complete individual urine samples were obtained from each subject during the first 24 hours, and 24 hour pooled specimens were collected during the remaining days of the experiment. Three milliliter aliquots of all plasma, saliva, and urine collections were counted on the same day in a scintillation well counter to an accuracy of ± 2 per cent. Trichloroacetic acid (20 per cent) filtrates and precipitates of the plasma samples were also counted in the well counter. Determinations were not carried beyond 72 hours when the counting rates of the samples became too low to be determined accurately. The relative percentages of I^{131}-labeled substances (diiodotyrosine and iodide) present in the protein-free plasma filtrate, saliva, and urine samples were determined from the counting rates of individual spots observed on paper chromatograms. The chromatographic techniques and assay methods employed have been presented previously.[11]

RESULTS

Plasma and saliva concentrations of total radioactive iodine activity were plotted against time for the two groups of experimental subjects, and half-life values were calculated from the exponential portion of the respective disappearance curves (Table I, Column A). Cumulative urinary excretion curves of total radioactive iodine activity were also plotted, and excretion rate curves were prepared from these by plotting the percentage of the administered dose excreted

*Supplied by Abbott Laboratories. Average specific activity at the time of administration was 510 μc per milligram. The shipments were analyzed chromatographically, and 95 to 98 per cent of the radioactivity was present as diiodotyrosine. The sole radioactive contaminant appeared to be I^{131} iodide.

per unit time against time.[11, 12] Half-life values for urinary radioactive iodine excretion were interpolated from the latter curves, and these values are also summarized in Table I, Column A.

All of the I^{131} radioactivity observed on paper chromatograms of saliva specimens from control and experimental groups was found to be present as I^{131} iodide. However, chromatography of urine specimens from both groups revealed that significantly larger amounts of I^{131}-labeled diiodotyrosine were excreted by patients with liver disease (Table I, Column B). Essentially all of the diiodotyrosine in urine was excreted within the first 8 to 10 hours.

TABLE I. I^{131}-LABELED DIIODOTYROSINE TURNOVER DATA OBTAINED FOR SUBJECTS WITH AND WITHOUT LIVER DISEASE

	A T ½ VALUES			B I^{131} EXCRETED AS DIT IN	C PLASMA DIT TURNOVER DATA		D I^{131} TISSUE SPACE	E RENAL EXCRETION RATE
PATIENT NO.	PLASMA (HR.)	SALIVA (HR.)	URINE (HR.)	URINE (% DOSE)	T ½ (HR.)	K_L ($HR.^{-1}$)	(ML./KG.)	($r \times Q_f$) (% D/HR.)
Liver Disease.—								
1	17	17	19	15.5	1.9	1.01	510	3.1
2	13	13	12	13.7	1.4	0.73	400	3.8
3	13	13	15	17.6	0.8	0.80	690	3.4
4	22	19	15	14.5	0.8	0.73	550	3.1
5	16	16	11	5.9	1.2	1.13	570	1.9
6	9	12	16	*	1.1	0.98	480	3.6
7	12	11	17	13.1	0.8	1.17	450	3.6
8	21	28	†	†	1.5	0.90	740	†
9	11	10	10	15.0	4.5	0.46	750	4.6
Average	14.9	15.4	14.4	13.6	1.6	0.88	571	3.4
Control Subjects.—								
10	9	9	7	4.8	1.3	0.83	260	6.1
11	13	11	†	†	5.2	0.50	510	†
12	8	8	10	1.4	3.2	0.65	470	3.2
13	8	8	6	7.7	1.0	0.87	620	4.2
14	9	8	9	2.3	2.2	0.46	430	7.5
15	8	8	8	1.0	2.0	0.97	570	4.4
16	10	10	9	7.6	1.4	1.29	640	5.5
17	11	10	10	6.0	0.6	0.93	690	2.2
Average	9.5	9.0	8.4	4.4	2.1	0.81	525	5.1

*Insufficient radioactivity on chromatograms for accurate counting.
†Calculations unavailable because of incomplete urine collections.

Since diiodotyrosine does not bind to plasma proteins, the plasma proteins were precipitated with 20 per cent trichloroacetic acid, and the resulting filtrates were chromatographed on paper. Only two radioactive spots appeared on the chromatograms, and these were identified through their R_f values as iodide ion and diiodotyrosine. The two radioactive spots were counted, and the per cent of the administered dose of I^{131} present at each spot was calculated. These values obtained for each specimen of plasma were plotted against time to yield I^{131} diiodotyrosine disappearance and I^{131} iodide appearance curves (Fig. 1). The diiodotyrosine disappearance curves obtained from both experimental groups were found to fit the expression $P_t = C_1e^{-m_1t} + C_2e^{-m_2t}$ which was derived in a previous investigation.[11] P_t represents the concentration of diiodotyrosine in the plasma at any time t, C_1 is the intercept of slope m_1 on the

ordinate, and C_2 is the intercept of slope m_2 on the ordinate. Half-life values for diiodotyrosine disappearance are summarized in Table I, Column C, together with the calculated deiodination rate constants K_L. The latter were calculated[11] from the expression $K_L = \frac{m_2 - m_1}{\sqrt{5}}$, and represent the rate with which diiodotyrosine is deiodinated, presumably by the liver (L). If tissues other than the liver are actually responsible for diiodotyrosine deiodination, then K_L may be considered as the deiodination rate constant for the tissues involved.

DISCUSSION

Contrary to our initial hypothesis, no significant differences in the average diiodotyrosine disappearance times (T ½) and diiodotyrosine deiodination rates (K_L) were observed between patients with liver disease and control subjects (Table I, Column C). However, a small but significantly greater amount of diiodotyrosine was excreted into the urine of persons with liver disease (Table I, Column B); this observation correlates with that of Dunn and co-workers[13] who found an increased excretion of certain amino acids such as tyrosine into the urine of patients with liver disease. On the basis of these findings, it would appear that the liver may not be all-important for the deiodination of diiodotyrosine, and that liver disease in the human does not have an appreciable effect upon the rate of diiodotyrosine deiodination. On the other hand, a significant amount of parenchymatous liver disease may be present without producing significant abnormalities in other tests of liver function, and it is probable that sufficient functioning liver tissue remained in the subjects studied to enable diiodotyrosine catabolism to proceed at a near normal rate. Other investigators[14, 15] have also suggested that the liver may not be the most important site of diiodotyrosine deiodination, whereas it may be very important for the deiodination or catabolism of thyroxine and triiodothyronine. Flock and associates[14, 15] have shown that the rate of diiodotyrosine deiodination is not reduced in the eviscerated rat nor in the hepatectomized dog, but that the deiodination of thyroxine and triiodothyronine is markedly affected. More recently, Becker and Prudden[16] have shown that the rates with which thyroxine and triiodothyronine are metabolized by an isolated rabbit liver preparation are approximately six times those seen in the intact animal. The deiodination of diiodotyrosine by the same liver preparation proceeds at approximately the same rate as that observed in the intact animal. These experimental data indicate that the diiodotyrosine deiodination mechanism probably is not confined solely to the liver.

Paper chromatographic separation of the I^{131}-labeled substances present in plasma (Fig. 1) and urine specimens revealed that intravenously administered diiodotyrosine is deiodinated very rapidly as shown by others.[1-3] Approximately 10 hours after injection 80 to 90 per cent of the circulating I^{131} activity is present as the iodide. Furthermore, the concentration of I^{131}-labeled diiodotyrosine falls so rapidly that the plasma I^{131} iodide curve approaches and becomes nearly equal to that of the whole plasma I^{131} disappearance curve (Fig. 1). Therefore, a few hours after injection of I^{131}-labeled diiodotyrosine, the

study becomes resolved into one of the effects of liver disease upon I^{131} iodide metabolism, and it would appear from the data presented in Table I, Column A, that liver disease does affect the disappearance rate of I^{131} iodide from the body as evidenced by prolonged plasma, saliva, and urine half-life values. These values were obtained from the final exponential portions of the respective disappearance curves, and consequently represent close approximations of the disappearance rate of I^{131} iodide from the body. At least three explanations for this effect of hepatic disease upon I^{131} iodide disappearance might be considered. First, if the uptake of I^{131} iodide by the thyroid gland was depressed in liver disease, the disappearance of I^{131} iodide from the plasma might take place at a slower rate. Mueller and co-workers[17] have found that the converse is frequently true in that I^{131} thyroidal uptake was 50 per cent higher in 20 of 50 patients with cirrhosis. It has been suggested that this increased uptake may be due to dietary iodine deficiency in patients with cirrhosis.[18] In view of these observations, it does not appear that the prolonged half-life disappearance values observed in the present experiment can be explained by a depressed thyroidal uptake of I^{131}.

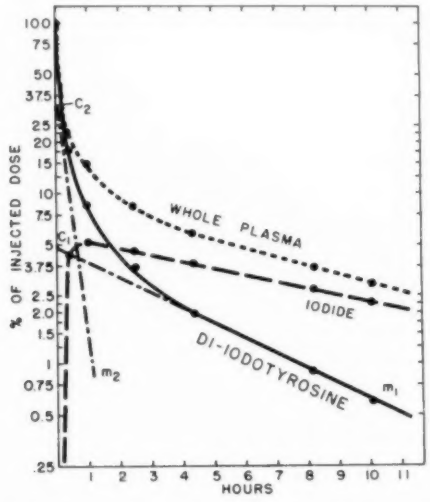

Fig. 1.—Typical whole plasma I^{131} activity disappearance, plasma I^{131}-labeled iodide appearance, and diiodotyrosine disappearance curves. The latter were obtained by chromatographic resolution of the I^{131}-labeled components which appeared in plasma.

A second explanation might be that the tissue space available for radioactive iodine diffusion was reduced in size, thus resulting in a decreased disappearance rate of I^{131} iodide from plasma. The increased edema and ascites often seen in liver disease would tend to discount this possibility, but as an added check, I^{131} tissue spaces* were estimated for the two groups of patients

*Estimates of the tissue space available for I^{131} diffusion were obtained by dividing the counting rate for the dose by the counting rate per 1 ml. of plasma at zero time. The latter value was obtained by extrapolating the final slope of the whole plasma I^{131} disappearance curve back to the ordinate, and reading the value from the ordinate at the intersection. Tissue space values are then expressed as milliliters per kilogram of body weight.

(Table I, Column D). These data show no significant differences between the two groups, and lead to the conclusion that another mechanism must be responsible for the prolonged I^{131} iodide disappearance rate seen in liver disease.

Finally, if the renal excretion of I^{131} iodide activity was decreased in patients with liver disease, this might maintain the concentration of I^{131} iodide in plasma at a higher level and thus produce a decreased I^{131} iodide disappearance rate from the plasma and other tissues. There is ample evidence from the literature[19, 20] that abnormal kidney function is frequently present in patients with liver disease (cirrhosis). These abnormalities, which involve the excretion and tubular reabsorption of water and electrolytes, are apparently a function of the severity of the liver disease encountered. To examine this possibility as an explanation for our data, renal excretion rates of iodide were calculated from the cumulative urinary excretion curves, according to the method of Keating and collaborators.[21] These calculations, which are summarized in Table I, Column E, show that the rate of renal excretion of I^{131} iodide is significantly decreased in liver disease. Therefore, abnormal kidney function associated with the condition of hepatic disease may account for a decreased disappearance of I^{131} iodide from plasma and other tissues.

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EARLY DETECTION OF DRUG INDUCED ERYTHROPOIETIC DEPRESSION

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An increase in plasma iron content and an increase in the saturation of iron binding globulin precedes the fall in hematocrit by an appreciable interval in erythropoietic depression induced by chloramphenicol and a related drug. Although serial changes in plasma iron content are not in themselves conclusive evidence of erythropoietic depression, there is an excellent correlation between these determinations and the more definitive studies with Fe^{59} turnover by erythrocytes. Accordingly, a rise in the plasma iron content and an increase in saturation of the iron binding globulin can serve as a reliable and sensitive index of early erythropoietic toxicity. This would permit the discontinuance of a potentially noxious drug before changes become apparent in the peripheral blood. It is suggested that routine serial determinations of plasma iron content be performed in conjunction with the administration of drugs with potential hematologic toxicity in an attempt to avoid serious injury to the marrow.

Since the nitrobenzene moiety of chloramphenicol has been implicated in drug toxicity, the effect on drug toxicity of substituting a $-CH_3SO_2$ radical for the NO_2 group in chloramphenicol was studied. A marked increase in erythropoietic inhibition resulted from this substitution which suggests that this particular position in the structural configuration may be of importance in hematologic toxicity.

MANY therapeutic agents now commonly employed are known to have potentially serious hematologic effects. However, the continued clinical application of these drugs is justified on the grounds that their therapeutic efficacy outweighs their potential toxicity. The introduction of new drugs presents difficulty because compounds may have unsuspected hematologic toxicity which becomes evident only after extensive clinical trial. Currently, hematologic toxicity is determined by careful observation of serial changes in the peripheral blood counts. This has the disadvantage of permitting withdrawal of the offending agent only after obvious hematopoietic depression has occurred, which in some instances may be either irreversible or sufficiently severe to contribute to the death of seriously ill patients.

The earliest possible detection of drug induced hematopoietic depression is desirable in order to permit the prompt discontinuance of drug therapy before potentially irreversible damage occurs. A simple screening procedure which

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would permit such early detection of hematopoietic damage might be a valuable aid in decreasing the incidence of severe and possibly fatal hematologic reactions. Data from previous studies on chloramphenicol toxicity suggest that changes in iron metabolism may be a very sensitive index of *erythropoietic* depression. Such changes were demonstrated to occur before abnormalities were detectable in the peripheral blood counts.[1] These studies included measurement of the rate of disappearance of tracer amounts of radioactive iron (Fe^{59}) from the plasma, determination of the subsequent appearance of Fe^{59} in the circulating erythrocytes, and changes in the plasma iron content and plasma iron binding globulin saturation. A decrease in the rate at which Fe^{59} disappeared from the plasma and a delay in the appearance of Fe^{59} in the circulating erythrocytes was noted as an early finding in patients who subsequently developed an appreciable anemia. Thus, the Fe^{59} data were clearly abnormal at a time when the hemoglobin, hematocrit, and erythrocyte counts were still at normal levels. It was also noted that a rise in plasma iron content and an increasing saturation of the iron binding globulin could be correlated with the abnormalities detected in the more technically difficult and time consuming radioactive iron determinations. Serial determinations of the plasma iron and saturation of the iron binding globulin may therefore constitute a highly sensitive method of screening for incipient drug induced *erythropoietic* depression.

It is not known why some drugs are more likely to produce hematologic toxicity than others. The presence of certain radicals or chemical combinations may be involved but the structural configuration responsible for a relatively high incidence of hematologic toxicity in any drug has not been clearly elucidated. In the case of chloramphenicol, the nitrobenzene moiety of the molecule has been implicated as a possible factor in producing damage to the bone marrow.[2-5] Chloramphenicol is an effective agent against a number of resistant microbial infections in man and its use may be essential at times despite its known hematologic toxicity. Although the absolute incidence of such toxicity is low, the reactions may be serious and often fatal.[5-7, 11, 12] A drug having a similar spectrum of antibacterial action without the hematologic toxicity which occurs with chloramphenicol would have an obvious advantage. An antibiotic having the same range of effectiveness as chloramphenicol, and with a similar structural configuration, was described in 1952.[8] This compound, dextrosulphenidol, differs from chloramphenicol only in that a methylsulfone group replaces the nitro group in the para position (Fig. 1). Such structural alteration could result in less hematopoietic depression than chloramphenicol causes if the nitrobenzene moiety is, indeed, responsible for the observed toxicity.

The following study was undertaken to determine whether ferrokinetic studies would be useful in determining the potential hematologic toxicity of this antibacterial agent (dextrosulphenidol) and to compare its toxicity with that of chloramphenicol. The study was also designed to determine the validity of the preliminary observations[1] that serial changes in plasma iron content and iron binding globulin saturation could be correlated with the more definitive Fe^{59} turnover by erythrocytes as an early index of erythropoietic depression.

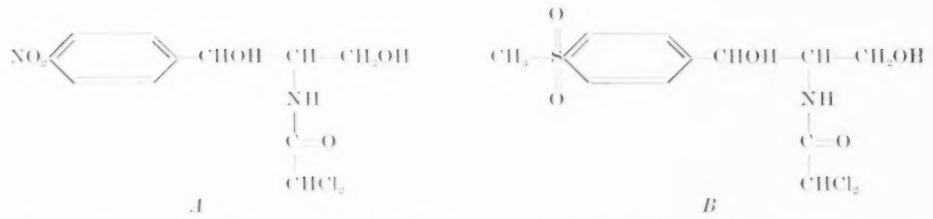

Fig. 1.—*A*, Chloramphenicol: threo-2-dichloroacetamido-1-(p-nitrophenyl)-1, 3-propanediol. *B*, Dextrosulphenidol: threo-2-dichloroacetamido-1-(4-Methylsulfonylphenyl)-1, 3-propanediol.

MATERIALS AND METHODS

Ninety-seven healthy men, volunteers from the State Penitentiary, McAlister, Oklahoma, participated in the study. Forty-nine of these individuals were given 1.0 Gm. of chloramphenicol twice daily for 28 days. Forty-eight of the volunteers were given 1.0 Gm. of dextrosulphenidol twice daily. In five individuals who were receiving dextrosulphenidol the drug was discontinued in 10 days because of a rise in plasma iron content and increasing saturation of the iron binding globulin; in another five the drug was discontinued within 15 days because of delayed plasma clearance of Fe^{59} as well as changes in plasma iron, and in nine subjects it was discontinued in 3 weeks because of a decreasing hematocrit as well as changes in plasma iron and delayed Fe^{59} clearance. The remaining 29 subjects received dextrosulphenidol for a full 28 days.

Routine hematologic studies were performed in duplicate during the week, before the study period. Hemoglobin determinations were performed with a Sheard-Sanford photolometer using a 1:400 dilution. Packed red cell volume was determined from venous blood, using heparinized capillary tubes and centrifuging for 5 minutes in an International microhematocrit centrifuge. Leukocyte counts were performed using 0.5 per cent acetic acid as a diluent in standard pipettes. Differential leukocyte counts were obtained by the wet method using brilliant cresyl blue for staining. Platelet counts were performed by the direct method using Rees-Ecker diluent and were the only determinations not obtained in duplicate during the control period. All determinations were repeated once weekly during the period of drug administration and for 2 weeks after the study was discontinued.

Plasma iron and iron binding globulin capacity were determined in all subjects by the method of Schade and associates.[9] Blood was drawn when the subjects were in a fasting state between 4:30 and 5:30 A.M. Duplicate values were obtained prior to the period of drug ingestion and were repeated at weekly intervals for 4 weeks thereafter.

The rate of removal of Fe^{59} from the plasma (expressed as half-life disappearance or T/2) and its subsequent incorporation into erythrocytes was determined by a modification of the methods of Huff and coauthors,[10] which was previously described.[1] These studies were performed on twenty-eight subjects who were receiving dextrosulphenidol and thirteen subjects who were receiving chloramphenicol. In most instances an attempt was made to select for study those subjects who gave some indication of an erythropoietic effect as shown by an increase in saturation of the iron binding globulin. Radioactive iron in the form of ferrous citrate* was diluted with 5 per cent dextrose in water so that 20 ml. contained approximately 10 μc of Fe^{59} (5 to 10 mcg. of elemental iron) and was injected intravenously. Incubation of the Fe^{59} with the subject's plasma prior to injection was not carried out since the total circulating unsaturated iron binding globulin is in excess of the amount of iron administered and the injected iron is bound rapidly in vivo. Heparinized blood samples were obtained at 5, 15, 30, 60, and 90 minutes after injection of the Fe^{59} from the opposite arm and the plasma was removed for counting in a well-type scintillation counter.† Subsequently,

*Fe^{59} was supplied by Abbott Laboratories in the form of ferrous citrate, 100 μc per milliliter and 48 mcg. per milliliter.

†NRD Instrument Co., Model Cs-600

the radioactivity appearing in the circulating erythrocytes was determined by counting 1 ml. whole blood 24 hours after injection of Fe^{59} and daily thereafter until 100 per cent incorporation was obtained.

RESULTS

Using the criteria outlined in Table I the subjects were classified as exhibiting either definite erythropoietic depression, borderline erythropoietic depression, or no significant depression. These criteria were chosen as the basis

TABLE I. CRITERIA FOR ERYTHROPOIETIC DEPRESSION

DETERMINATION	NORMAL* VALUES	DEFINITE ERYTHROPOIETIC DEPRESSION†	BORDERLINE ERYTHROPOIETIC DEPRESSION‡
Hematocrit	46 ± 3.0%	A decrease of 5.5% or >	A decrease of 3-5.4%
Serum iron	90 ± 35 mcg. %	An increase of 120 mcg. % or >	An increase of 55-119 mcg. %
IBG§ saturation	30 ± 10%	An increase of 45% or >	An increase of 20-44%
Fe^{59} T/2	50-100 min.	120 min. or >	110-120 min.
Fe^{59} appearance in RBC			
Initial (days)	2	3 or >	3
Maximum (days)	8	13 or >	8-12
Maximum (%)	100	85 or <	85-90

Increase refers to an absolute rise over control values. Thus an increase of 120 mcg. per cent in serum iron would represent a rise from 90 to 210 mcg. per cent. An increase of 45 per cent in the iron binding globulin saturation would represent an increase in saturation from 30 to 75 per cent.

*Normal: insignificant change in any parameter.

†Definite erythropoietic depression: abnormal in all parameters studied.

‡Borderline erythropoietic depression: abnormal in two or more parameters but not in all.

§IBG: iron binding globulin.

for classification by correlating delayed Fe^{59} turnover by erythrocytes and fall in hematocrit on the one hand with prolongation of Fe^{59} plasma disappearance, rise in plasma iron content, and increasing saturation of iron binding globulin on the other hand. Except for three of the five subjects in whom dextrosulphenidol was discontinued within 10 days, only those who demonstrated abnormalities in all parameters studied were classified as definitely manifesting erythropoietic depression. In these three subjects erythropoietic depression was determined on the basis of delay in the reappearance of Fe^{59} in circulating erythrocytes, prolongation of Fe^{59} plasma disappearance, and increasing iron binding globulin saturation, even though the hematocrit did not fall below the arbitrary level chosen as indicating toxicity. Presumably the fall in hematocrit did not take place because the drug was discontinued within 10 days. Subjects were classified as exhibiting borderline erythropoietic depression if they had abnormalities in two or more of the parameters studied but did not have abnormal values in all parameters. Those subjects in whom insignificant changes occurred were classified as manifesting no erythropoietic depression.

According to these criteria, 32 (66 per cent) of those who were receiving dextrosulphenidol had evidence of definite erythropoietic depression, seven (15 per cent) had borderline erythropoietic depression, and nine (19 per cent) had

no significant toxicity (Table II). In comparison, only one (2 per cent) of the subjects who were receiving chloramphenicol manifested definite erythropoietic toxicity; three (6 per cent) had borderline erythropoietic toxicity, and forty-five (92 per cent) had no significant erythropoietic depression. The average maximum changes occurring in hematocrit, plasma iron content, saturation of iron binding globulin, and incorporation of Fe^{59} into erythrocytes during the ingestion of dextrosulphenidol are summarized in Table III. Serial changes occurring in the hematocrit, plasma iron, and saturation of iron binding globulin in the thirty-two subjects who developed erythropoietic depression during dextrosulphenidol ingestion are compared to those in nine subjects without erythropoietic toxicity in Fig. 2. These demonstrate that an increase in the plasma iron content and a rise in the saturation of the iron binding globulin precedes a significant fall in the hematocrit by a 2 week period.

The average values for the plasma iron content and saturation of the iron binding globulin in subjects who exhibited erythropoietic toxicity with dextrosulphenidol are compared with those obtained during the control period in Fig. 3. The delay in Fe^{59} clearance (half-life) from the plasma in a subject who developed erythropoietic toxicity while ingesting dextrosulphenidol and the return to a normal value after the drug was discontinued are shown in

TABLE II. COMPARISON OF INCIDENCE OF ERYTHROPOIETIC DEPRESSION WITH CHLORAMPHENICOL AND DEXTROSULPHENIDOL

	CHLORAMPHENICOL		DEXTROSULPHENIDOL	
	NO.	%	NO.	%
Definite erythropoietic depression	1	2	32	66
Borderline erythropoietic depression	3	6	7	15
No erythropoietic depression	45	92	9	19

TABLE III. SUMMARY OF DATA: PATIENTS RECEIVING DEXTROSULPHENIDOL

DETERMINATION	CONTROL VALUES (49 SUBJECTS)	AVERAGE MAXIMUM CHANGES DURING TEST PERIOD		
		DEFINITE ERYTHROPOIETIC DEPRESSION	BORDERLINE ERYTHROPOIETIC DEPRESSION	NO ERYTHROPOIETIC DEPRESSION
Hematocrit (%)	46.9 ± 2.8	− 8.1	− 5.0	− 2.7
Serum iron (mcg. %)	86.3 ± 33	+136	+110	+59
TIBC saturation (%)	29.6 ± 11	+ 47	+ 36.6	+20
Fe^{59} T/2 (min.)	—	>406	117	75
Fe^{59} reappearance in erythrocytes				
Initial (days)	—	9.8*	3†	2‡
Maximum (days)	—	>18*	13†	8‡
Maximum (%)	—	62*	87†	99‡

*Data from 22 subjects.
†Data from 2 subjects.
‡Data from 2 subjects.

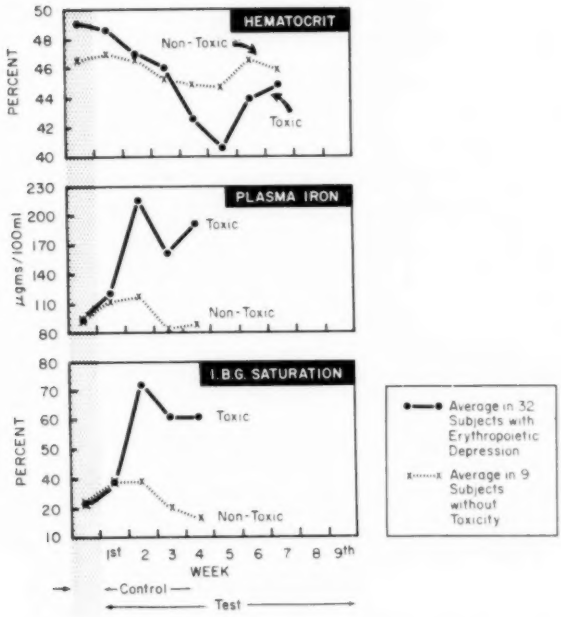

Fig. 2.—Serial changes in hematocrit, plasma iron, and iron binding globulin (*I.B.G.*) saturation during ingestion of a chloramphenicol derivative (dextrosulphenidol). The values in subjects who developed erythropoietic depression are compared with those in subjects without toxicity. The changes in plasma iron and iron binding globulin saturation precede a significant fall in hematocrit by a 2 week period.

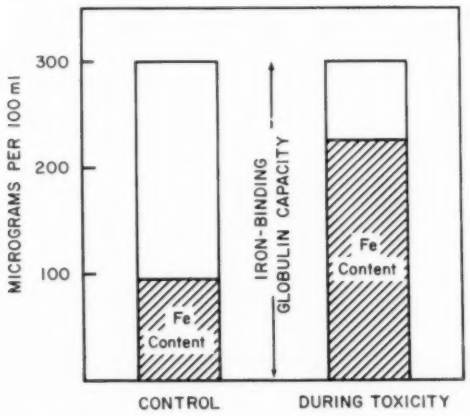

Fig. 3.—Changes in plasma iron content and saturation of iron binding globulin in subjects who developed erythropoietic depression during the ingestion of dextrosulphenidol. The increase in plasma iron and increase in saturation of iron binding globulin occur as an early manifestation or erythropoietic depression and correlate with delayed incorporation of radioactive iron into circulating erythrocytes and subsequent fall in hematocrit.

Fig. 4. The delay in reappearance of radioactivity in the circulating erythrocytes in a subject who developed erythropoietic toxicity is contrasted to the normal reappearance in a subject without toxicity in Fig. 5.

No permanent erythropoietic depression developed in any of the subjects and the hematocrit returned to normal in every instance by the time the study was discontinued (Fig. 2). None of the subjects exhibited any significant alteration in the leukocyte or platelet count during the period of the study. Except for the transient erythropoietic depression, no other toxic manifestations were noted in patients who were receiving either chloramphenicol or dextrosulphenidol.

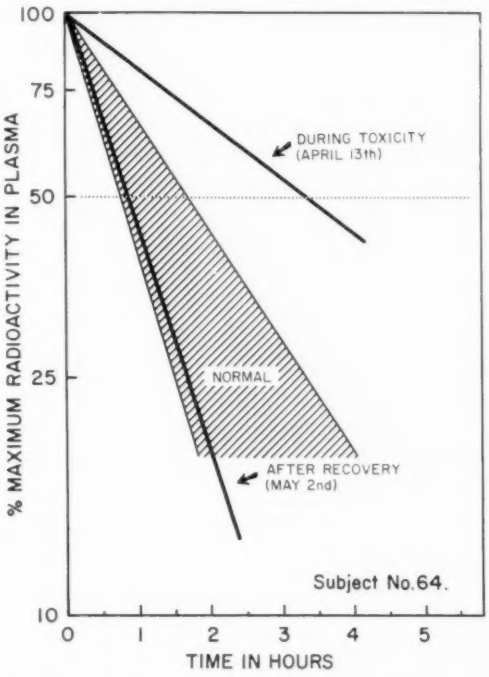

Fig. 1.—Plasma clearance of Fe^{59} during and after erythropoietic depression associated with dextrosulphenidol ingestion. The delay in Fe^{59} clearance which develops as a result of toxicity is compared with a normal value in the same subject after ingestion of the drug was discontinued.

DISCUSSION

The data demonstrate an excellent correlation between increasing plasma iron content, increasing saturation of iron binding globulin, prolonged plasma clearance of Fe^{59}, delay in reappearance of Fe^{59} in circulating erythrocytes, and fall in hematocrit as tests for erythropoietic depression. With the exception of Fe^{59} turnover by erythrocytes, none of the ferrokinetic determinations are in themselves proof of erythropoietic inhibition. However, in every instance where Fe^{59} plasma clearance was delayed, there was a subsequent delay in reappearance of Fe^{59} in the circulating erythrocytes. Furthermore, in all but three instances, an increase in plasma iron content and rise in saturation of iron binding globulin was also correlated with delay in reappearance of Fe^{59} in erythrocytes. It is particularly important to note that the rise in plasma iron

content and increased saturation of iron binding globulin preceded the fall in hematocrit by a significant interval. Since a delay in the reappearance of Fe^{59} in erythrocytes is the most reliable ferrokinetic index of erythropoietic depression and since there is such an excellent correlation of this delay with changes in the plasma iron, serial changes in plasma iron content and iron binding globulin saturation can serve as a sensitive and reliable screening procedure for early detection of *erythropoietic* depression. The excellent correlation between the changes in plasma iron and the more definitive tests for erythropoietic depression obviates the necessity for performing the more involved and time consuming studies with Fe^{59}. These determinations may be particularly useful since the changes occur at a time when the peripheral blood is still normal.

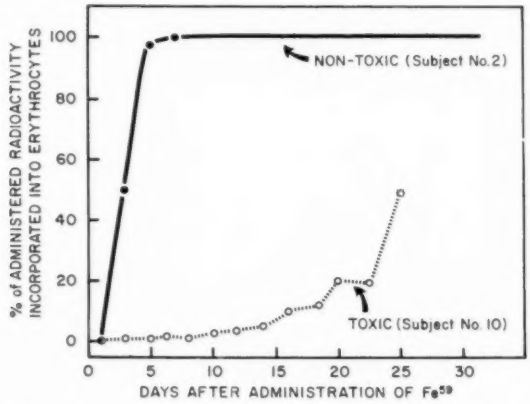

Fig. 5.—A comparison of the rate of appearance of Fe^{59} in circulating erythrocytes in a toxic and nontoxic subject. Both subjects received dextrosulphenidol. The subject without toxicity had 100 per cent of the Fe^{59} in the erythrocytes within 8 days. The subject who developed erythropoietic depression had only 55 per cent of the Fe^{59} in the erythrocytes after 25 days even though the drug was discontinued on the sixth day.

Erythropoietic depression was the only hematologic toxicity detected with both chloramphenicol and dextrosulphenidol in this study. Leukopenia, thrombocytopenia, and marrow aplasia occur as manifestations of chloramphenicol toxicity but the incidence of these serious hematologic reactions is thought to be low.[11, 12] Erythropoietic depression which occurs without other signs of marrow damage has seldom been reported as a manifestation of chloramphenicol toxicity. The preceding study on changes in iron metabolism in connection with chloramphenicol administration,[1] as well as the observations in this report, indicate that erythropoietic depression in itself is not a rare occurrence with either chloramphenicol or the related compound, dextrosulphenidol. Similar studies on other therapeutic agents might therefore be of interest.

Although erythropoietic depression may not necessarily be correlated with, or be followed by, other toxic manifestations such as leukopenia, thrombocytopenia, and aplastic anemia, it is possible that continued use of a drug which produces erythropoietic depression might eventually result in a general marrow depression. Because of the life span of the erythrocyte, erythropoietic depression could exist for some time without an appreciable fall in peripheral blood

counts being noted, thereby allowing the continued use of a potentially dangerous drug. Such an assumption is unproved and it remains to be determined whether early discontinuance of drug therapy in patients manifesting erythroid depression will result in a decreased incidence of other hematologic toxicity. It would seem desirable, however, to perform routine serial determinations of plasma iron content in conjunction with the administration of drugs with potential hematologic toxicity. Such routine determinations might aid in increasing the safety with which drugs are administered.

Although erythropoietic depression was relatively uncommon with chloramphenicol, it was a frequent occurrence with dextrosulphenidol. Under the conditions of this study the toxic manifestations were mild and innocuous. In all instances, the erythropoietic depression was reversible after the drug was discontinued. However, the incidence of toxicity in this report was determined in healthy volunteers and may not reflect either the incidence or the type of toxicity which might occur in seriously ill individuals. Factors such as infection, associated impairment of renal or hepatic function, and dosage and duration of therapy may affect the incidence as well as type of toxicity which may be encountered.

Substitution of a $-CH_3SO_2$ radical for the NO_2 group in the nitrobenzene moiety of chloramphenicol apparently results in the marked increase of erythropoietic toxicity of dextrosulphenidol as compared to that of chloramphenicol. This particular position in the structural configuration may therefore be vital in the mechanism by which these compounds exert hematologic toxicity. Further studies on the effect of the substitution of other radicals in this position on antibacterial spectrum and hematologic toxicity are indicated.

SUMMARY AND CONCLUSION

An increase in plasma iron content and an increase in the saturation of iron binding globulin preceded the fall in hematocrit by an appreciable interval in drug induced erythropoietic depression. Although serial changes in plasma iron content are not in themselves conclusive evidence of erythropoietic depression, there is an excellent correlation between these determinations and the more definitive studies with Fe^{59} turnover by erythrocytes. Accordingly, a rise in the plasma iron content and an increase in saturation of the iron binding globulin can serve as a reliable and sensitive index of early erythropoietic toxicity. This would permit the discontinuance of a potentially noxious drug before changes become apparent in the peripheral blood. It is suggested that routine serial determinations of plasma iron content be performed in conjunction with the administration of drugs with potential hematologic toxicity in an attempt to avoid serious injury to the marrow.

Application of these determinations has been useful in the evaluation of the toxicity of an antibiotic with a chemical structure and antibacterial spectrum similar to those of chloramphenicol. Substitution of a $-CH_3SO_2$ radical for the NO_2 group in the nitrobenzene moiety of chloramphenicol results in a marked

increase in erythropoietic inhibition. This particular position in the structural configuration may therefore be vital in the mechanism by which these compounds exert hematologic toxicity.

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INFLUENCE OF SERUM PROTEIN BINDING ON RENAL CLEARANCE OF I^{131}-LABELED DIODRAST

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WITH THE TECHNICAL ASSISTANCE OF VALENTINE BIKERMAN AND DOROTHY GRAHAM

The recent clinical use of I^{131}-labeled Diodrast in evaluating renal function has prompted the study of its renal clearance in man. Clearances were found to be low at plasma concentration of I^{131}-labeled Diodrast below 1 mcg. per cent whereas, at higher plasma concentrations, clearances were normal. It was also shown that plasma protein binding of I^{131}-labeled Diodrast occurred at all concentrations studied and was increased at levels below 1 mcg. per cent. Unlabeled Diodrast saturated plasma protein binding sites for I^{131}-labeled Diodrast and the simultaneous administration of unlabeled Diodrast resulted in normal clearances of I^{131}-labeled Diodrast at all plasma concentrations. These results were interpreted as demonstrating plasma protein binding to be a limiting factor in the renal excretion of I^{131}-labeled Diodrast at the low plasma concentrations achieved with the labeled compound.

DIODRAST (iodopyracet), initially used for measurements of renal plasma flow and tubular excretory capacity, was replaced by para-aminohippurate because of technical difficulties in its measurement.[2] The recent availability of I^{131}-labeled Diodrast has led to its use for evaluating renal function both by external monitoring techniques[3-5] and in physiologic studies.[6] There have been, however, no studies in man concerned with the renal clearance of such minute quantities of Diodrast as are administered as the tagged compound.

This report demonstrates that the clearance of I^{131}-labeled Diodrast is depressed at low plasma concentrations and may be restored to normal with "carrier" or stable Diodrast, presumably through the effects of carrier Diodrast on plasma protein binding of I^{131}-labeled Diodrast.

MATERIALS AND METHODS

Standard clearance techniques were employed in 20 men without known cardiovascular or renal disease. One hour after a single injection of I^{131}-labeled Diodrast, renal clearance ($C_{V/P}$) of the residual I^{131}-labeled Diodrast was calculated using interpolated values

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to correct for the slight fall in plasma levels observed at this time. In other studies, renal clearance of I^{131}-labeled Diodrast was performed, using a prime and constant infusion of I^{131}-labeled Diodrast, at plasma concentrations of less than 2 μg per cent. In several experiments, stable Diodrast was also administered, and in two subjects studied, the simultaneous clearance of I^{131}-labeled Diodrast and stable Diodrast was determined. Urine samples were obtained by having the patient void, plasma samples by venipunctures using heparinized syringes. All compounds were administered intravenously.

Equilibrium dialysis experiments were carried out on freshly drawn serum to which had been added varying quantities of I^{131}-labeled Diodrast alone or in combination with stable Diodrast. Samples were placed in Cellophane bags (36 by 32 Dextar) and dialyzed against twenty times the plasma volume of isotonic phosphate buffer at pH 7.3. Equilibrium was usually established within 24 hours; however, dialysis was continued for 48 hours at 5° C. Some samples of serum were repeatedly dialyzed against fresh buffer and the ratio of plasma radioactivity to buffer radioactivity was successively obtained. No significant binding of I^{131}-labeled Diodrast to the Cellophane bags was observed.

Radioactivity of samples containing I^{131}-labeled Diodrast was measured in a well-type NaI(T1) scintillation counter. Chemical concentration of I^{131}-labeled Diodrast was calculated from the specific activity of the dose used. Chemical concentration of stable Diodrast was determined by iodine analysis using a modified Barker distillation-digestion method.[7]

The I^{131}-labeled Diodrast* used for these studies was subjected to ascending paper chromatography using a solvent of glacial acetic acid, butanol, and water (10:75:15) and descending paper chromatography using phenol and 20 per cent hydrochloric acid, with a barbital buffer. Strips were placed on x-ray film and exposed for 1 to 2 weeks; they were also sectioned and counted to estimate the percentage of impurities. Two impurities were found along with I^{131}-labeled Diodrast in the commercial product. Impurity "A" had a Rf of 0.18; impurity "B" had a Rf of 0.86 in the ascending system. The amounts of impurity were always less than 1.0 per cent of the total radioactivity present. These impurities were not noted on chromatography of the 30 minute urine specimen obtained after administration of I^{131}-labeled Diodrast. Twenty microcuries of I^{131}-labeled Diodrast was usually given as the single dose. One gram of stable Diodrast was used when carrier was given unless otherwise noted.

RESULTS

Renal Clearance and Extraction Ratios of I^{131}-Labeled Diodrast.—One hour after an injection of I^{131}-labeled Diodrast alone, concentrations of I^{131}-labeled

TABLE I

SUBJECT	TIME (MIN.)	URINE FLOW (ML./MIN.)	PLASMA I^{131}-LABELED DIODRAST (μG %)	CLEARANCE I^{131}-LABELED DIODRAST (ML./MIN.)
Sar	1	Inject: 0.15 mg. I^{131}-labeled Diodrast, I.V.		
	45	Urine: discard		
	61	7.8	0.17	210.5
	86	9.2	0.16	185
	116	11.8	0.14	190.9
Ros	1	Inject: 1.2 mg. I^{131}-labeled Diodrast, I.V.		
	45	Urine: discard		
	60	14.1	0.17	261
	80	13.3	0.14	275
	115	12.8	0.11	243.2
Leh	1	Inject: 0.18 mg. I^{131}-labeled Diodrast, I.V.		
	57	Urine: discard		
	122	7.1	0.17	161.5

*Abbott Laboratories, Oak Ridge, Tenn.

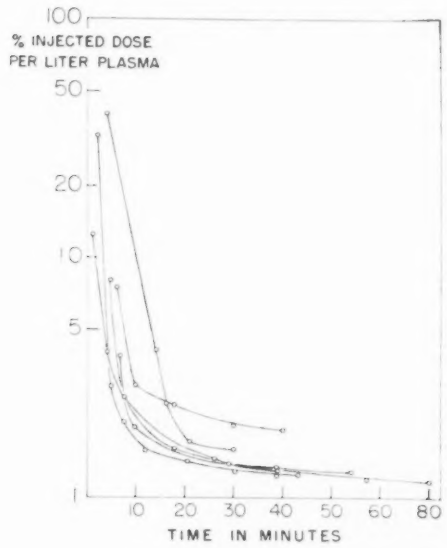

Fig. 1.—Plasma disappearance of I^{131}-labeled Diodrast. Plateauing of plasma I^{131}-labeled Diodrast concentration was observed within the first 30 minutes after injection.

Diodrast in plasma decreased slowly in the plateau phase (Fig. 1). Clearances performed at such levels ranged from 185 to 275 ml. per minute, averaging 218 ml. per minute. (Table I). In a patient undergoing right heart catheterization for evaluation of mitral stenosis, blood samples were obtained from the right renal vein after an injection of I^{131}-labeled Diodrast.* The renal extraction ratio for I^{131}-labeled Diodrast determined during the plateau phase of the plasma I^{131}-labeled Diodrast curve was found to be 0.51. In another patient undergoing renal exploration† with ether anesthesia, direct sampling of the right renal artery and vein revealed that the I^{131}-labeled Diodrast extraction ratio was 0.85 during the rapid fall-off and 0.52 during the plateau phase of plasma radioactivity. When carrier Diodrast was administered simultaneously with I^{131}-labeled Diodrast, abrupt plateauing of plasma radioactivity was not observed; the plasma I^{131}-labeled Diodrast curve resembled plasma stable Diodrast curve (Fig. 2).

Effects of Carrier Diodrast on the Renal Clearance of I^{131}-labeled Diodrast.—With constant infusion techniques, it was shown that the calculated clearance of I^{131}-labeled Diodrast was related to its plasma concentration at low levels. Below I^{131}-labeled Diodrast plasma concentrations of 0.5 μg per cent, clearances were less than 340 ml. per minute. Above I^{131}-labeled Diodrast concentrations of 0.8 μg per cent, clearances were more than 500 ml. per minute. In some subjects clearances were serially raised to normal levels by raising the plasma concentration of I^{131}-labeled Diodrast (*Sar, Blo, Gor*, Fig. 3.) Differences in urine flow induced by mannitol diuresis did not influence calculated clearances (*Blo,*

*Kindness of Dr. William Hollander.
†Kindness of Dr. George P. Whitelaw.

TABLE II

SUBJECT	TIME (MIN.)	URINE FLOW (ML./MIN.)	PLASMA I^{131}-LABELED DIODRAST (μG %)	CLEARANCE I^{131}-LABELED DIODRAST (ML./MIN.)
Leh	1	Prime: 0.5 mg. I^{131}-labeled Diodrast, I.V.		
	2	Infuse I^{131}-labeled Diodrast at 14.1 mcg. per minute to end		
	51	Urine: discard		
	66	1.0	1.6	646.9
	82	2.2	1.7	740
	83	Infuse stable Diodrast at 40 mg. per minute		
	98	5.6	2.2	409
	114	12.2	2.3	590.9
	115	Inject: 7 Gm. stable Diodrast, I.V.		
	116	Increase rate of stable Diodrast infusion 260 mg. per minute		
	134	9.4	2.5	315
	150	13.1	4.0	262
Sar	1	Prime: 0.39 mg. I^{131}-labeled Diodrast, I.V.		
	2	Infuse I^{131}-labeled Diodrast at 2.4 mcg. per minute		
	111	Urine: discard		
	126	1.3	0.72	381.5
	150	3.6	0.71	382.5
	165	8.2	0.79	353
	182	12.7	0.61	324.8
	198	13.6	0.59	334.4
	199	Infuse stable Diodrast at 11.6 mg. per minute to end		
	215	12.1	0.64	278.2
	231	12.6	0.65	312
	246	13.7	0.65	333.3
	262	14.1	0.64	363.7
Ash	1	Prime: 0.17 mg. I^{131}-labeled Diodrast and 0.87 Gm. of stable Diodrast		
	2	Infuse I^{131}-labeled Diodrast at 5.4 mcg. per minute and stable Diodrast at 10.8 mg. per minute		
	32	Urine: discard		
	47	10.2	0.45	709
	63	11.4	0.31	639
	81	11.3	0.25	517
	96	12.9	0.22	587
	111	14.6	0.17	559

Sol, Fig. 3). The clearance of I^{131}-labeled Diodrast was decreased by doses of stable Diodrast which exceeded anticipated rates of maximum tubular secretory capacity (Leh, Table II).

At established low clearance rates of I^{131}-labeled Diodrast, the addition of carrier Diodrast to a constant infusion of I^{131}-labeled Diodrast did not increase the clearance of I^{131}-labeled Diodrast during the period of observation (Sar, Table II). However, when carrier Diodrast was administered as a prime along with I^{131}-labeled Diodrast, and both were constantly infused in similar proportions, normal clearances of I^{131}-labeled Diodrast were obtained at low plasma concentrations of I^{131}-labeled Diodrast (Ash, Table II, and Bab, Table III).

In subject Bab, the clearance of stable Diodrast averaged 7.9 per cent higher than the I^{131}-labeled Diodrast clearance. In a similar study where the prime of stable Diodrast was omitted, marked differences in clearance of I^{131}-labeled Diodrast and carrier Diodrast were seen (Gor, Table III). In this instance, clearance of carrier Diodrast averaged 38.4 per cent higher than the clearance of I^{131}-labeled Diodrast.

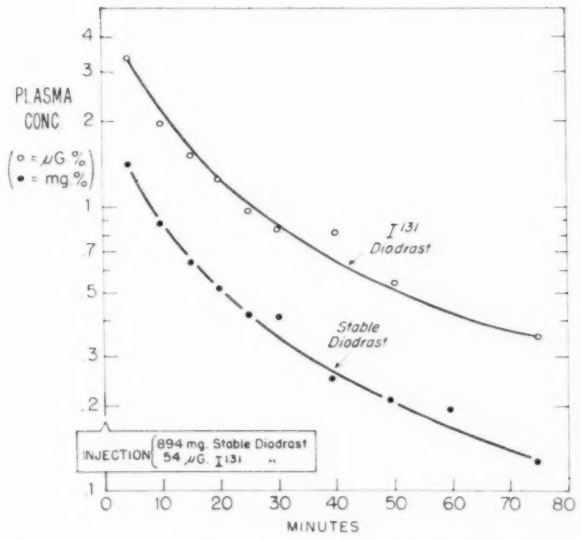

Fig. 2.—Simultaneous plasma disappearance of stable Diodrast and I^{131}-labeled Diodrast. When the compounds were administered together plateauing of plasma I^{131}-labeled Diodrast was not seen for 30 minutes after injection.

Equilibrium Dialysis Studies of Serum Protein Binding of I^{131}-Labeled Diodrast.—Using the ratio of serum I^{131}-labeled Diodrast activity to buffer I^{131}-labeled Diodrast activity as an index of serum protein binding, it was found

TABLE III

SUBJECT	TIME (MIN.)	URINE FLOW (ML./MIN.)	PLASMA CONCENTRATION		CLEARANCE	
			I^{131}-LABELED DIODRAST (μG%)	STABLE DIODRAST (MG.%)	I^{131}-LABELED DIODRAST (ML./MIN.)	STABLE DIODRAST (ML./MIN.)
Bab	1	Prime: 85 mcg. I^{131}-labeled Diodrast and 310 mg. stable Diodrast				
	2	Infuse I^{131}-labeled Diodrast at 0.5 mcg. per minute and stable Diodrast at 1.7 mg. per minute				
	90	Urine: discard				
	105	9.3	0.17	0.125	495	500.4
	120	7.4	0.17	0.115	388	538
	135	7.6	0.17	0.105	460	559
	150	7.3	0.16	0.105	366	440.5
	151	Increase infusion to 1.5 mcg. per minute I^{131}-labeled Diodrast and 5.1 mg. per minute stable Diodrast				
	166	Urine: discard				
	182	7.3	0.75	0.50	478	542.9
	197	8.1	0.72	0.50	685	715.6
	212	5.9	0.74	0.55	489	498.7
[illegible]or	1	Prime: 877 mcg. I^{131}-labeled Diodrast				
	2	Infuse I^{131}-labeled Diodrast at 1.6 mcg. per minute and stable Diodrast at 11.02 mg. per minute				
	61	Urine: discard				
	75	15.4	0.66	5.5	471	724.6
	91	15.8	0.56	4.7	392	681.7
	106	14.3	0.49	4.4	335	560.3
	115	16.4	0.59	4.8	385	619.1

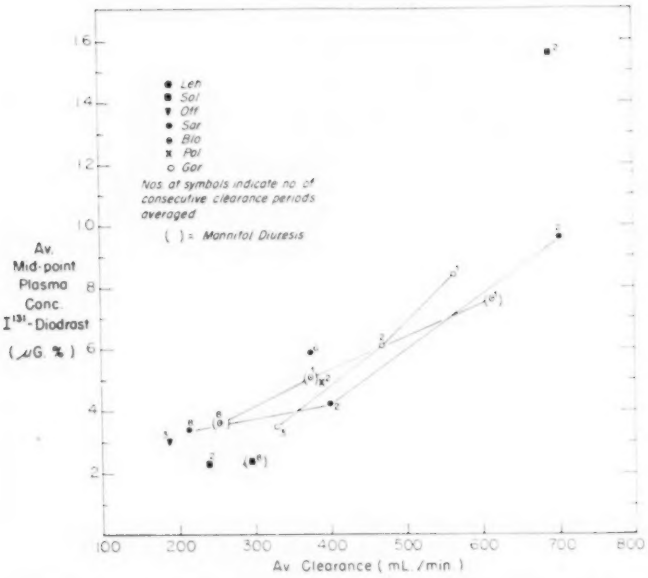

Fig. 3.—Influence of plasma concentration on clearance of I^{131}-labeled Diodrast. Between plasma concentrations of 0.4 and 0.8 μg per cent of I^{131}-labeled Diodrast, marked changes in renal clearance was observed. Low clearance rates were unaffected by mannitol diuresis.

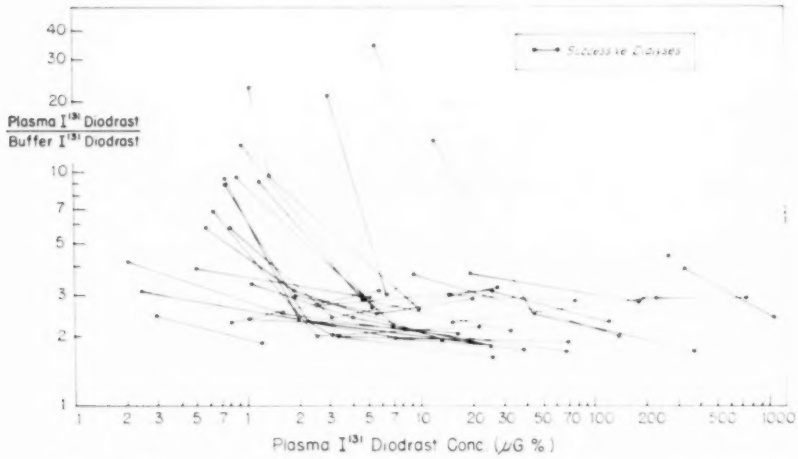

Fig. 4.—Plasma protein binding of I^{131}-labeled Diodrast. With successive dialyses, increased plasma to buffer ratios were most regularly obtained below plasma I^{131}-labeled Diodrast concentrations of 2 μg per cent.

that binding of I^{131}-labeled Diodrast occurred at all concentrations tested. Successive dialysis permitted evaluation of binding in a single serum sample with a one thousand fold change in concentration of I^{131}-labeled Diodrast. At serum concentrations from 100 to 1,000 μg per cent, the serum to buffer ratio averaged 2.8. At serum concentrations from 10 to 100 μg per cent, the ratio also averaged

2.8. At serum concentrations from 2 to 10 μg per cent, the ratio averaged 3.5. Below serum concentrations of 2 μg per cent, the ratio averaged 6.1 (Fig. 4). When I^{131}-labeled Diodrast (after dilution over a similar range) was added directly to serum, differences in binding were also observed at serum concentrations below 2 μg per cent. When I^{131}-labeled Diodrast was added to buffer rather than serum and dialyzed, no sequestration of I^{131}-labeled Diodrast within the bag was noted at the concentrations tested (Fig. 5).

Addition of carrier Diodrast to serum to achieve concentrations up to 0.5 Gm. per cent prior to addition of I^{131}-labeled Diodrast did not affect the ratios of 2 to 3 observed for I^{131}-labeled Diodrast. Higher serum concentrations resulted in lower serum to buffer ratios (Fig. 6). These larger amounts of carrier

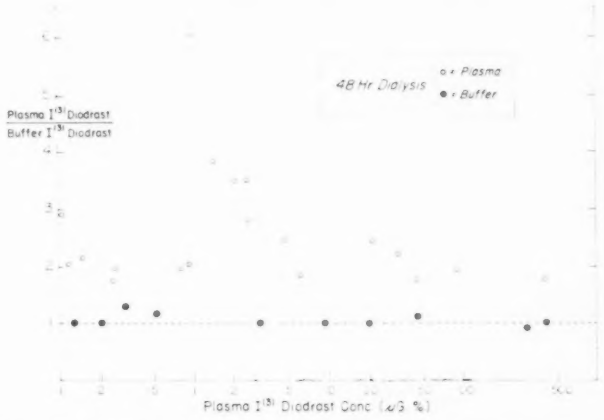

Fig. 5.—Influence of diffusion on ratios of plasma to buffer I^{131}-labeled Diodrast. When I^{131}-labeled Diodrast was added to buffer alone and dialyzed, diffusion was not a limiting factor in equilibration at 48 hours. When I^{131}-labeled Diodrast was added to plasma, highest ratios were observed at plasma concentrations of 1 to 2 μg per cent.

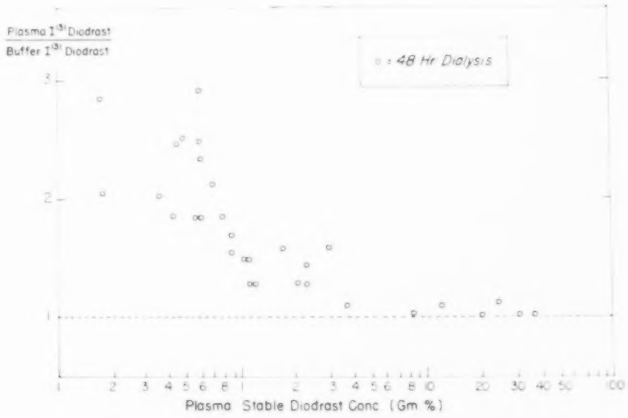

Fig. 6.—Saturation of plasma protein binding of I^{131}-labeled Diodrast by carrier Diodrast. Known amounts of stable Diodrast were added to plasma at the concentrations indicated before dialysis against buffer of 20 times the plasma volume.

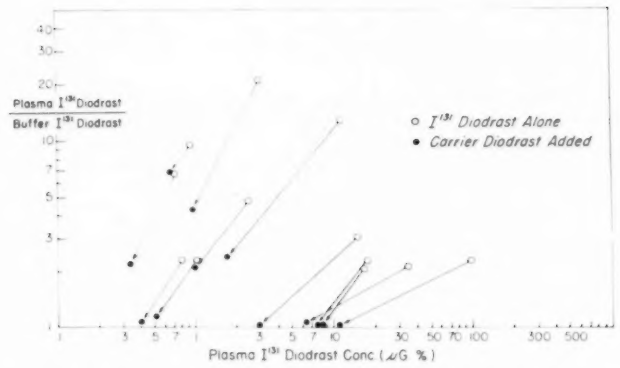

Fig. 7.—Displacement of I^{131}-labeled Diodrast from plasma proteins by carrier Diodrast. Displacement of I^{131}-labeled Diodrast was frequently incomplete below plasma concentrations of 2 μg per cent.

Diodrast were able to displace a part of the bound I^{131}-labeled Diodrast from the serum proteins at all concentrations of I^{131}-labeled Diodrast, but to a lesser extent at low serum concentration of I^{131}-labeled Diodrast (Fig. 7).

DISCUSSION

The renal excretion of Diodrast is so efficient that there is almost complete extraction from the plasma perfusing the renal tubules. Limitations of such transport across renal tubular cells have been ascribed previously to cellular factors. Maximum tubular secretion of Diodrast has been shown to occur when plasma concentration is raised to saturate an intracellular "carrier transport" system.[8] At lower plasma levels, calculated clearances are said to be independent of plasma concentrations.[9] In the present study, the renal excretion of I^{131}-labeled Diodrast was found to be dependent on plasma concentration at extremely low plasma levels of the chemical compound.

Using both a single injection technique and sustained infusions of I^{131}-labeled Diodrast, calculated clearances of I^{131}-labeled Diodrast were found to be much below anticipated normal levels for Diodrast of 700 ml. per minute.[9] Also, during the rapid plasma fall-off phase of I^{131}-labeled Diodrast given alone, the observed renal extraction ratio of 0.85 was comparable to the extraction ratio of 0.94 reported for para-aminohippurate, but the extraction ratio of 0.54 observed during the plateau phase was not. The decreased excretion of I^{131}-labeled Diodrast at low plasma levels may be explained in several ways: (1) a portion of the administered material is impure and cleared at a slower rate than I^{131}-labeled Diodrast; (2) at low plasma concentrations a significant proportion of I^{131}-labeled Diodrast is reabsorbed by the tubules; and (3) as plasma concentration falls, I^{131}-labeled Diodrast binding by plasma proteins becomes a limiting factor in the rate of tubular secretion. Combinations of these possibilities may exist.

Chromatographic separation of commercial I^{131}-labeled Diodrast reveals several impurities which do not appear in the urine, but these apparently occur in concentrations that are too small to be responsible for the present findings.

In addition, if the plateau phase seen with a single injection of I^{131}-labeled Diodrast were entirely a manifestation of such impurities, it might not be affected by the administration of stable Diodrast, whereas in fact it was essentially abolished. Also, low clearance rates were obtained with a constant infusion of I^{131}-labeled Diodrast at low plasma levels.

Such established low clearance rates were not altered by the subsequent addition of relatively large amounts of carrier Diodrast. The two substances were cleared at different rates, which would rule out tubular reabsorption as an explanation for the low I^{131}-labeled Diodrast clearances. Tubular reabsorption has not been described in man for Diodrast, para-aminohippurate, or phenol red, although reabsorption of I^{131}-labeled Diodrast has been demonstrated in Necturus.[10] A priming dose and a sustaining infusion of carrier Diodrast with the I^{131}-labeled Diodrast did result in normal clearances of both substances at low plasma levels of I^{131}-labeled Diodrast.

A plasma protein saturation phenomenon is suggested by these observations. Using equilibrium dialysis, a relatively nondialyzable complex of plasma protein with I^{131}-labeled Diodrast was noted at plasma concentrations below 1 to 2 μg per cent, which coincided with the approximate range of plasma concentrations associated with decreased clearances of I^{131}-labeled Diodrast. At these low concentrations large amounts of carrier Diodrast were unable to completely displace the more firmly bound I^{131}-labeled Diodrast. Previous studies of plasma protein binding of Diodrast, using relatively high concentrations, have not demonstrated such differences in binding.[11] However, different binding affinities have been ascribed to different plasma proteins with corticosteroids,[12] thyroxin,[13] cholanic acid,[14] and vitamin B_{12},[15] probably representing a common biologic phenomenon.

Different plasma proteins with differing affinities for I^{131}-labeled Diodrast may be responsible for the present observations; several loci of binding on the same plasma protein with differing avidity for I^{131}-labeled Diodrast may also be an important factor.[16] Protein binding has been demonstrated to be of prime importance in the excretion of phenol red[17] and hemoglobin[18] and is responsible for the biologic half-life of 2.5 years for Teridax.[19] The protein-I^{131}-labeled Diodrast complex is apparently less dissociable at low plasma concentrations, resulting in less I^{131}-labeled Diodrast being extracted from the plasma perfusing the renal tubules. It is assumed that such interactions between tubular cells and the protein-I^{131}-labeled Diodrast complex occur at the cell surface, rather than within the renal tubular cell, although these observations do not rule out the occurrence of intracellular binding phenomenon.[20] It is not felt that these observations represent an isotope effect resulting from the labeling of Diodrast by I^{131} because of the small change in weight which results. Analytic techniques, sufficiently sensitive for such low plasma concentrations of stable Diodrast, are not available to confirm this belief.

SUMMARY

1. The renal clearance of I^{131}-labeled Diodrast is depressed at the low serum concentrations attained when I^{131}-labeled Diodrast is given alone.

2. The administration of carrier Diodrast restores the renal clearance of I^{131}-labeled Diodrast to normal levels.

3. Serum protein binding of I^{131}-labeled Diodrast at low serum concentrations can be demonstrated by dialysis techniques; this can be prevented by the addition of carrier Diodrast.

4. The depressed renal clearance of I^{131}-labeled Diodrast, given alone, may be due in part to its binding by serum proteins.

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LABORATORY METHODS

A RAPID AND SIMPLE METHOD FOR MEASURING THE RATE OF CEREBRAL BLOOD FLOW IN HUMANS WITH KRYPTON^{85}

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A simple procedure in which Krypton^{85}, a radioactive gas, is utilized was developed for measuring the rate of cerebral blood flow. Cerebral tissue is rapidly saturated with krypton^{85} by rebreathing the gas in a closed circuit for 2 minutes. At the termination of the saturation period, the subject is allowed to breathe air and eliminate krypton^{85} from the system. Cerebrovenous blood samples are drawn at one minute intervals over a period of 5 minutes during the elimination period. The rate of krypton^{85} elimination from cerebral tissue is measured by plotting the values of krypton^{85} concentration in blood samples—concentration versus time. The slope of the curve of elimination is indicative of the rate of cerebral blood flow expressed in milliliters per minute per 100 grams brain.

Krypton^{85} is extracted from blood samples and relative concentrations are measured in a Geiger-Müller gas flow counter.

Results obtained with this technique compared favorably with results obtained with the standard nitrous oxide method.

The advantages of utilizing a radioactive gas for measuring the rate of cerebral blood flow are: measurements are specific, simple, and rapid. Moreover, anesthetic gases do not interfere with the procedure of gas analysis.

LASSEN and Munck[1] have recently described a method for the determination of the rate of cerebral blood flow in human subjects in which Kr^{85} is substituted for nitrous oxide. The subject inhales a constant mixture of Kr^{85} in air in a closed ventilation system for 14 minutes; arterial and cerebral venous blood samples are withdrawn simultaneously at specific time intervals during this period. A total of 16 blood samples are collected and analyzed in special cuvettes designed to measure the relative blood concentrations of Kr^{85}. The major advantage of Kr^{85} over nitrous oxide is presumably a technical one, in that it permits specific, accurate, and rapid analysis of gas concentrations. The values obtained for cerebral blood flow in normal subjects are essentially the same as those reported by others who used nitrous oxide.

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In this preliminary report, the rate of total cerebral blood flow was determined in human subjects with Kr^{85}; the procedure of Lassen and Munck was modified and considerably simplified to permit rapid bedside determination of cerebral hemodynamics. Comparative values for total cerebral blood flow determined simultaneously with the modified Kr^{85} procedure and the nitrous oxide technique of Scheinberg and Stead are presented.

METHOD AND MATERIAL

Needles are inserted into the internal jugular bulb and femoral artery for blood sampling as originally described by Kety and Schmidt.[2] A dampened aneroid manometer is attached to the arterial manifold for monitoring mean arterial pressure.[3]

A carbon dioxide absorption unit, commonly used on anesthetic gas machines was modified so that it could be readily converted into a unidirectional, nonrebreathing system (Fig. 1). Two 2-way stopcocks, *A* and *B* (internal diameter ¾ inch) are placed in the gas flow circuit. With both stopcocks in position *a* a closed ventilation system is established by introducing a rebreathing bag and soda lime cannister into the circuit. When stopcocks

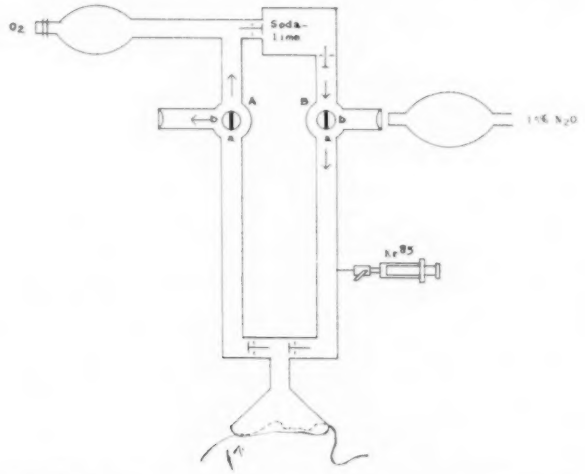

Fig. 1.—Rebreathing system for partial saturation of the central nervous system.

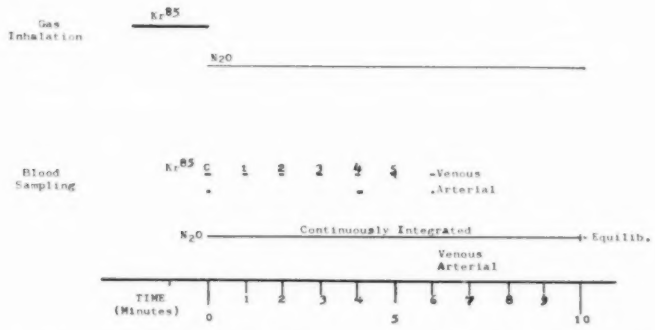

Fig. 2.—Sequence of blood sampling for simultaneous Kr^{85} and nitrous oxide techniques to determine the rate of cerebral blood flow.

A and *B* are turned to position *b*, a unidirectional nonrebreathing system is achieved with the inlet at stopcock *B* and outlet at stopcock *A*. Directional flutter valves placed in the face-mask adapter minimizes rebreathing and insures unidirectional flow of gases.

With stopcocks *A* and *B* placed in position *b*, the reservoir bag is filled with air and the face mask is firmly fixed over the subject's face, stopcock *A* and *B* are turned to position *a* (closed ventilation system) and 400 μc of Kr^{85} in air is injected into the reservoir bag. Oxygen (250 ml. per minute) is constantly supplied, for metabolic requirements, through a nipple in the reservoir bag. The subject is permitted to rebreathe the Kr^{85}-air mixture for $1\frac{1}{2}$ to 2 minutes (krypton saturation time); at the end of this period, stopcocks *A* and *B* are turned to position *b* (nonbreathing system) and 15 per cent N_2O, 21 per cent O_2, and 69 per cent N_2 is introduced through the reservoir bag attached to inlet of stopcock *B*. This phase is designated as the Kr^{85} elimination period or desaturation phase and the beginning of the nitrous oxide inhalation or saturation period.

Two arterial and six cerebral venous blood samples are required for the determination of the rate of cerebral blood flow using Kr^{85}. Each blood sample (3 ml. accurately measured in 10 ml. heparinized syringe) is withdrawn within 5 seconds. The first arterial and cerebral venous blood samples are drawn simultaneously at the termination of the Kr^{85} saturation period. Five additional cerebrovenous samples are drawn at exactly one minute intervals thereafter, during the krypton elimination period. A second arterial blood sample (for background level determination) is drawn at the fourth minute of the Kr^{85} elimination phase (Fig. 2). All syringes are labeled and immediately sealed with metal caps to prevent loss of Kr^{85} gas from the blood samples.

Arterial and cerebral venous blood samples are simultaneously withdrawn at a specific rate when the subject commences the inhalation of the N_2O gas mixture over a 10 minute period, according to the procedure described by Scheinberg and Stead.[4] The withdrawal of cerebral venous blood for Kr^{85} determination is accomplished by inserting a syringe into the manifold; the brief interval required for the withdrawal of blood specimens does not appreciably influence the collection of blood for N_2O determinations. The cerebral and arterial blood samples are analyzed volumetrically for N_2O, CO_2, and O_2 contents and the rate of cerebral blood flow is calculated.

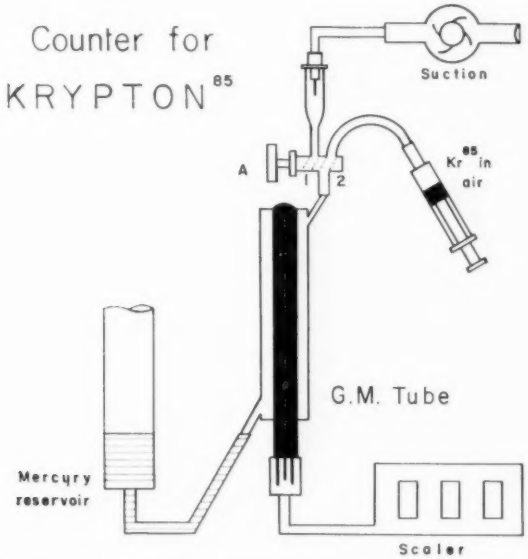

Fig. 3.—Gas flow Geiger-Müller detector for counting Kr^{85} in air.

Procedures for Measuring Concentration of Kr^{85} in Blood Samples.—Extraction of Kr^{85} from the blood samples is accomplished as follows: The barrel of the sealed syringe is withdrawn to the 10 ml. mark and held firmly in position while the metal cap is removed to permit the inflow of air. The cap is then replaced and the contents of the syringe (7 ml. air and 3 ml. blood) are vigorously shaken for 10 to 15 seconds; the syringes are then placed in a test tube rack, tip up, for 10 to 15 minutes.

A standard thin glass wall Geiger-Müller (M-4) gas flow detector is used for counting Kr^{85} (Fig. 3). A directional stopcock *(A)* has been adapted to the inlet opening and a reservoir which contains 20 to 30 ml. mercury to the outlet opening *(B)*. The mercury in the reservoir is adjusted to level off at the outlet opening at atmospheric pressure. The counting chamber is filled with mercury by applying suction to inlet stopcock *(A)* at position *1*. The inlet is then closed and the syringe which contains air and blood for Kr^{85} analysis is attached to stopcock *(A)* at *2*. Stopcock *(A)* is turned to position *2* and 6 ml. of gas is introduced into the counting chamber as the mercury level falls. The syringe is detached and as soon as the mercury level stabilizes and atmospheric pressure is reestablished, stopcock *(A)* is turned to seal off the inlet and the gas content is counted. After each analysis, the counting chamber is decontaminated by flushing it with air and spot-checks for atmospheric background levels are made between each analysis to insure proper decontamination.

Calculations.—The formula derived for the calculation of cerebral blood flow by means of Kr^{85} gas is based on observations made on the relative concentrations in arterial and cerebral venous blood at specific time intervals after cessation of rebreathing the radioactive gas (Fig. 4).

During the first minute, t_o to t_1 (immediately after the saturation period) arterial concentration *(a)* of Kr^{85} fall-off is extremely rapid and thereafter remains at varying but extremely low levels (Fig. 5).

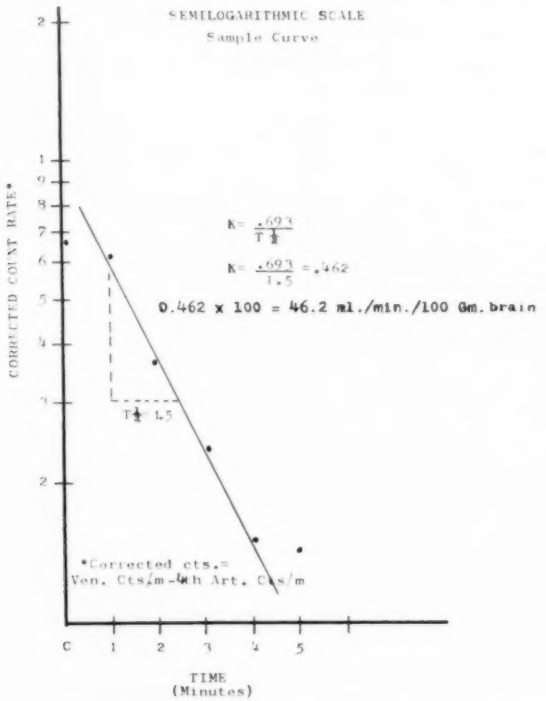

Fig. 4.—Method for calculating cerebral blood flow by the rate of elimination of Kr^{85} in cerebrovenous blood samples.

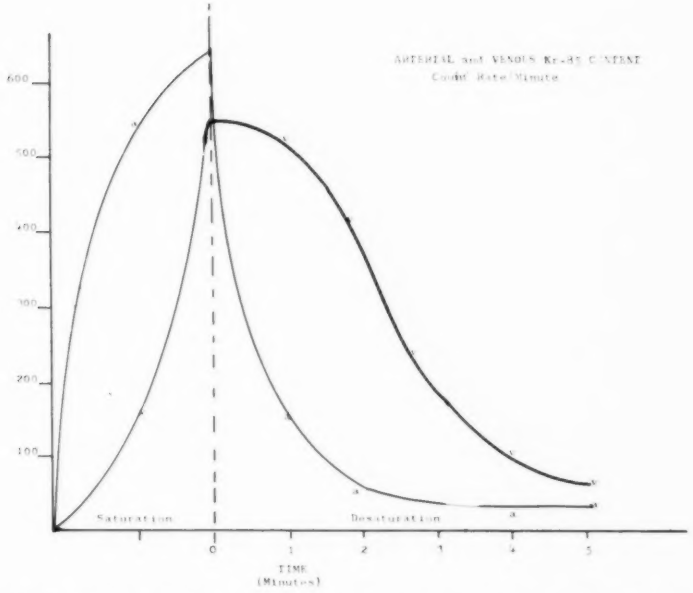

Fig. 5.—Curves obtained during rapid partial saturation of the central nervous system with Kr^{85} and behavior during the desaturation period.

Under normal conditions, with no disturbances, in Kr^{85} elimination (adequate pulmonary gaseous exchange) during the period t = 1 to t = 4, the low arterial content of Kr^{85} may be considered to constitute a slowly varying background concentration. Repeated experiments have shown that arterial concentration (a) of Kr^{85} content at the fourth minute during the elimination period is a representative measure of the mean arterial background concentration (G) for the time interval t = 1 to t = 4.

The fall-off of Kr^{85} in cerebral venous blood (v) during the period t_0 to t_1 suggests that there is no significant continued diffusion of the gas across to the brain after the saturation phase is completed. After this period, the arterial to venous contamination is minimal, to the extent of the variable low background (G). Concentration of Kr^{85} in cerebral venous blood during time interval t_1 to t_4 may be taken to indicate the diffusion of Kr^{85} to the blood from that accumulated in cerebral tissue. The rate of Kr^{85} fall-off in cerebral venous blood corrected for arterial background (v - G) may, after the first minute, be represented by an exponential curve for the time interval t = 1 to t = 4.

The formula for calculating rate of total cerebral blood flow is derived as follows:

$$\frac{dBM}{dt} = -Fv - Fa = -F(v-a) \qquad (1)$$

where M = mass of brain; B = concentration of Kr^{85} in brain tissue; v = concentration of Kr^{85} in cerebral venous blood; a = concentration of Kr^{85} in arterial blood; and F = blood flow through brain.

From the observations noted on the behavior of Kr^{85}, the following assumptions and substitutions were made:

a = G = a at t_4 background level for the period t_1 to t_4.
B = v = Cerebral venous concentration of Kr^{85} after t = 1.

Solving the differential equation:

$$(v-G = (v-G)_{(t=1)} \; e^{\frac{-F}{M}(t-1)} \qquad (2)$$

$$\frac{F}{M} = \frac{-d\ln(v-G)}{dt} \quad \text{(after t = 1 minute)} \qquad (3)$$

Utilizing this formula the data are applied as follows: The fourth minute arterial count rate value is subtracted from each of the cerebral venous count rate values. Cerebral venous count rate values thus corrected for arterial background, are plotted on semilogarithmic paper as shown in Fig. 4 The best straight line is drawn between points t = 1 to t = 4 and the slope of this line is measured.

A convenient method to measure the slope of this line is to determine the time it takes for the corrected cerebral venous count rate to fall to half its value by a decay factor of 2. This time interval is expressed as t^{1}_{2}. In terms of this quantity:

$$\frac{F}{M} = \frac{-d\ln\ (v\text{-}G)}{dt} \tag{4}$$

$$\frac{F}{M} = \frac{d\ln\ 2}{dt^{1}_{2}} \tag{5}$$

$$\frac{F}{M} = \frac{0.693 \times 100}{t^{1}_{2}} = \text{milliliters per minute per 100 grams of brain.} \tag{6}$$

RESULTS

Table I presents total cerebral blood flow rates obtained using the Kr^{85} procedure described in 10 hospitalized patients ranging in age from 22 to 68 years. In order to determine the accuracy of the technique as well as to estimate the interval of time within which the procedure can be repeated, duplicate observations were obtained on each subject 5 minutes after the first flow was completed.

The mean value for total cerebral blood flow for both the first and second determinations reveals a finding of 47.91 ml. per 100 grams per minute for the former and 49.50 ml. per 100 grams per minute for the latter; the average difference is 1.37 ml. per 100 grams per minute with a standard deviation of 0.0691.

Table II presents the comparative data obtained using simultaneously the Scheinberg and Stead's nitrous oxide procedure and the Kr^{85} method presently under study. The 19 observations were obtained on 15 hospitalized patients ranging from 33 to 68 years in age and with no overt evidence of cerebral dysfunction. The mean value of 46.9 ml. per 100 grams per minute for total cerebral flow with the Kr^{85} technique is not significantly different from that (48.1 ml. per 100 grams per minute) obtained by the N_2O procedure. The ratio of blood flow value (Kr^{85} to N_2O) is 0.980 with a standard deviation of 0.067.

TABLE I. RESULTS OF DUPLICATE MEASUREMENTS OF RATE OF CEREBRAL BLOOD FLOW WITH Kr^{85} MODIFIED TECHNIQUE

CASE NO.	AGE (YR.)	DUPLICATED RESULTS		DIFFERENCES
		1	2	
1	63	47.9	46.1	1.8
2	46	42.9	41.3	1.6
3	41	52.5	53.7	1.2
4	68	58.2	64.2	6.0
5	48	44.5	42.2	2.3
6	54	46.2	50.2	4.0
7	53	46.7	48.0	1.3
8	33	53.9	58.8	4.9
9	50	37.3	38.0	0.7
10	22	49.0	52.6	3.6
Total		479.1	495.1	27.4
Mean		47.9	49.5	2.74

Mean average = 48.7. Average difference = 1.37. Average fraction difference = 2.81 per cent. Ratio between 1 and 2 = 0.9741. Standard deviation of differences = 0.0691.

TABLE II. COMPARISON OF RESULTS OBTAINED ON RATE OF CEREBRAL BLOOD FLOW MEASURED SIMULTANEOUSLY WITH Kr^{85} AND NITROUS OXIDE TECHNIQUES

CASE NO.	AGE (YR.)	Kr^{85}	N_2O	($Kr : N_2O$)
1	41	42.4	49.3	0.860
2	33	53.9	59.1	0.911
3	64	38.0	39.1	0.971
4	53	46.4	52.9	0.886
5	60	40.4	39.8	1.015
6	68	58.2	60.5	0.962
7	48	44.5	50.0	0.889
8	46	41.2	43.5	0.944
9	60	47.4	48.5	0.978
10	60	56.3	61.7	0.912
11	58	58.8	59.9	0.982
12	50	37.3	33.7	1.105
13	42	37.8 (right)	34.7	1.089
		41.7 (left)	42.1	0.992
14	53	50.9 (right)	45.7	1.112
		53.1 (left)	53.0	1.001
15	53	47.9 (right)	48.1	0.995
		47.9 (left)	46.1	1.050
		46.1 (left)	47.2	0.976
Total	19	890.2	914.9	18.630
Mean		46.9	48.1	

Ratio of cerebral blood flow values determined with Kr^{85} to the N_2O values was 0.980 with a standard deviation of 0.067.

In Table III data on 60 hospitalized patients ranging from 23 to 82 years of age with varied diagnoses are presented. In this group of subjects the rate of total cerebral blood flow was also determined simultaneously by both the Kr^{85} technique and the established N_2O procedure. The mean value for Kr^{85} of 39.26 is, again, not significantly different from that of 40.14 obtained using N_2O. The ratio of Kr^{85} to N_2O observed in these studies was 1.008 with a standard deviation of 0.1408. It is important to note that excellent agreement between the two procedures was obtained despite the fact that the individual rates of cerebral blood flow varied from 16.0 to 77.0 ml. per 100 grams per minute using the N_2O procedure.

DISCUSSION

A procedure to be applicable to clinical conditions should be reasonably simple to perform, reliable, and not too time consuming, and the results obtained must be accurate. Scheinberg and Stead modified the original nitrous oxide technique of Kety and Schmidt primarily to reduce the number of volumetric gas analyses. Lassen and Munck[1] subsequently substituted Kr^{85} for N_2O to further eliminate the necessity for gas analysis and presumably to improve the accuracy of the technique. Despite the above modifications, the presently available techniques for measuring cerebral blood flow are still somewhat cumbersome, time consuming and are not readily adaptable to clinical situations which necessitate rapid and repeated bedside evaluations.

The ventilation system described by Lassen and Munck for the administration of Kr^{85} is not only cumbersome and intricate to operate, but also presents the possibility of contamination. The use of a simple portable circle absorber system by which the isotope may be administered and collected, eliminates many

TABLE III. RESULTS OF RATE OF CEREBRAL BLOOD FLOW OBTAINED SIMULTANEOUSLY WITH Kr^{85} AND NITROUS OXIDE TECHNIQUES ON A SERIES OF 60 CASES WITH A VARIETY OF PATHOLOGIC CONDITIONS AND INDUCED PHYSIOLOGIC CHANGES

CASE NO.	PATIENT	AGE (YR.)	DIAGNOSIS	CLINICAL	Kr^{85}	N_2O	$Kr : N_2O$
1	MW	50	Alcoholism	Hypothermia	34.8	28.4	1.225
2	WA	59	Cancer of pancreas	Control	25.4	25.7	0.988
3	WA	59	Cancer of pancreas	Hypothermia	34.0	34.3	0.991
4	FM	46	Cardiac arrest	Control	28.3	30.3	0.934
5	BN	81	Cancer of rectum	Morphine	46.5	38.8	1.198
6	BN	81	Cancer of rectum	Hypothermia	39.8	35.4	1.124
7	BM	60	Cirrhosis, coma	Control	30.4	32.3	0.941
8	BM	60	Cirrhosis, coma	Argen.-Glut.	35.8	40.5	0.884
9	WA	24	Pneumonia	Control	35.8	37.5	0.955
10	WJ	65	Diabetes	Hypothermia	32.0	32.6	0.982
11	PH	48	Glioblastoma	Control	30.4	34.2	0.889
12	PH	48	Glioblastoma	Hypothermia	30.4	27.5	1.105
13	HK	36	Internal carotid thrombosis	Pre-Lig. RT.	46.5	46.8	0.994
14	HK	36	Internal carotid thrombosis	Pre-Lig. LT.	51.7	40.8	1.267
15	HK	36	Internal carotid thrombosis	Post-Lig. RT.	38.8	50.8	0.764
16	HK	36	Internal carotid thrombosis	Post-Lig. LT.	36.8	46.5	0.791
17	PH	48	Glioblastoma	Control	40.0	40.2	0.995
18	PH	48	Glioblastoma	Hypothermia	35.8	37.7	0.950
19	SM	59	Alcoholism	Control	34.0	36.6	0.929
20	RR	57	Cancer of colon	Control	21.0	27.0	0.778
21	RR	57	Cancer of colon	Levophed	26.0	28.9	0.900
22	MJ	55	Pancreatitis	Control	46.5	45.0	1.033
23	MJ	55	Pancreatitis	Hypothermia	46.5	51.7	0.890
24	KA	47	Cirrhosis	Coma	30.0	42.0	0.714
25	MJ	55	Pancreatitis	Control	73.0	77.0	0.948
26	MJ	55	Pancreatitis	Levophed	48.0	53.5	0.897
27	ER	47	Gastrointestinal malignancy	Control	69.0	67.2	1.027
28	ER	47	Gastrointestinal malignancy	Epinephrine	65.0	67.6	0.962
29	JM	78	Cancer of breast	Control	34.0	38.8	0.876
30	JM	78	Cancer of breast	Levophed	35.8	35.7	1.003
31	HM	82	Shock, pulmonary infarction	Control	34.8	34.6	1.006
32	HM	82	Shock, pulmonary infarction	Levophed	33.0	35.6	0.927
33	HK	36	Internal carotid thrombus	Right	58.0	50.6	1.146
34	HK	36	Internal carotid thrombus	Left	55.7	57.9	0.962
35	CC	69	Cardiovascular arrest, shock	Control	34.0	33.4	1.018
36	CC	69	Cardiovascular arrest, shock	Levophed	46.0	34.2	1.345
37	PG	66	Cancer of stomach	Levophed	65.0	62.4	1.042
38	MB	50	Pneumonia	Control	51.7	52.6	0.983
39	MB	50	Pneumonia	Levophed	42.2	41.8	1.010
40	PM	80	Cancer of stomach	Control	20.0	24.6	0.813
41	PM	80	Cancer of stomach	Epinephrine	31.0	28.6	1.084
42	AM	23	Pneumonia	Control	50.0	47.3	1.057
43	FM	46	Cardiac arrest	Control	35.8	48.3	0.741
44	BR	27	Pneumonia	Control	46.5	40.5	1.148
45	BR	27	Pneumonia	Epinephrine	51.7	52.9	0.977
46	TH	49	Pneumonia	Control	36.8	45.7	0.805
47	TH	49	Pneumonia	Epinephrine	20.0	27.0	0.740
48	PC	88	Generalized arteriosclerosis	Control	39.0	44.9	0.868
49	PC	88	Generalized arteriosclerosis	Epinephrine	39.8	45.0	0.884
50	WA	70	Arteriosclerotic heart disease	Control	35.8	40.0	0.895
51	WA	70	Arteriosclerotic heart disease	Epinephrine	41.0	38.9	1.054

TABLE III—CONT'D

CASE NO.	PATIENT	AGE (YR.)	DIAGNOSIS	CLINICAL	Kr^{85}	N_2O	Kr : N_2O
52	ME	53	Pneumonia	Control	55.7	52.0	1.071
53	GL	66	Arteriosclerotic heart disease	Nembutal	20.2	16.9	1.195
54	GL	66	Arteriosclerotic heart disease	Hypothermia	31.7	24.7	1.283
55	CM	75	Obstructive jaundice	Nembutal	22.5	18.1	1.243
56	CM	75	Obstructive jaundice	Hypothermia	16.0	17.0	0.941
57	EA	69	Syphilis	Control	41.0	42.6	0.962
58	EA	69	Syphilis	Phenothiazine	38.0	39.9	0.952
59	LA	74	Generalized arteriosclerosis	Control	45.0	38.5	1.169
60	LA	74	Generalized arteriosclerosis	Hypoglycemia	40.0	40.5	0.988
Total	60				2,355.8	2,408.3	60.467
Mean					39.26	40.14	

Ratio of cerebral blood flow values determined with Kr^{85} to the N_2O values was *1.008* with a standard deviation of *0.1408*.

of these objections; moreover, contamination is minimized since half the amount of Kr^{85} is used, and the rebreathing period is reduced from 14 to 2 minutes. Channeling the isotope to an outside vent for five minutes immediately after the saturation phase is completed further reduces room contamination. Where no outside vent is available the expired air can be collected in cellophane bags. The time required for the blood flow determination is significantly shortened and the number of blood samples collected for krypton counting has also been correspondingly reduced; the latter procedure has been greatly simplified.

Counting Kr^{85} in blood samples by Lassen's procedure entails the use of special cuvettes with thin mica windows, filled with blood under light pressure and sealed. The mica windows must be carefully calibrated as to thickness, since beta particles are nonpenetrating and only a superficial thin film of blood is measured. Counting cuvettes are placed between two balanced Geiger-Müller detectors and the highest count achieved is 7,000 counts per 15 minutes or 460 to 470 c.p.m. Inadvertent introduction of air in the cuvettes will give uneven distribution of Kr^{85} over the counting surface area. Total time required for counting the 16 specimens is 135 minutes. The material for counting Kr^{85} which we used is relatively inexpensive. The high count obtained by the technique described, of 3,000 counts per 2 minutes averaging 1,000 c.p.m. is approximately twice the count rate obtained with the cuvette system. The total time required for counting 7 specimens, which includes preparation of samples, counting time, manipulation, and a 15 minute equilibration time at room temperature, is 45 minutes.

The results obtained with the present procedure indicate that its accuracy and reliability have not been affected by the modifications described. The observations presented in Table I not only demonstrate the close agreement in values obtained on the same patient (same internal jugular vein) but also indicate that a relative saturation of the brain with Kr^{85} can be accomplished within 1½ to 2 minutes and that complete desaturation occurs within 5 minutes. Since the partition coefficients for N_2O and Kr^{85} are similar, 1.06 and 1.059,[5]

respectively, the latter observation should not be too surprising since practically complete saturation is achieved, in most instances, with N_2O within the first 3 minutes of its inhalation. The fact that with Kr^{85} repeat studies can be performed within 5 minutes indicates that in subjects with no pulmonary ventilation impairment the elimination of this gas from brain and blood proceeds at a rapid rate.

The close agreement in cerebral blood flow rates observed between results obtained using Kr^{85} desaturation and N_2O saturation procedures, as demonstrated in Table II, further indicates the reliability of the procedure and is evidenced also by the ratio of approximately unity attained on mathematical evaluation. The standard deviation of 0.1408 for the krypton procedure further reflects the accuracy of the present method when compared to that for the N_2O technique of 0.125.[2]

The close agreement between mean values observed for the Kr^{85} and N_2O procedures on subjects with cerebral blood flow rates well above and below normal values indicates its applicability for clinical studies since the saturation and desaturation time phases, as utilized, are sufficiently adequate for abnormal flow rate measurements.

Finally, if the results presented in this preliminary report using the Kr^{85} procedure are confirmed, important studies such as the effects of various anesthetic gases on cerebral hemodynamics and acute conditions with changes in rapid peripheral vascular resistance previously not possible with N_2O could now be obtained.

SUMMARY

A simple rapid method for measuring the rate of cerebral blood flow with Kr^{85} is described. Results presented suggest that this procedure described is as accurate as the standard nitrous oxide methods.

We wish to acknowledge the cooperation and technical assistance of William K. Young and Julia V. V. Johnson of the Metabolic Research Laboratory, and Miss Elenore Zekas, Anesthesiology Isotope Research Laboratory, District of Columbia General Hospital, and Talbot A. Chubb for developing methods of calculation and statistical analyses of data.

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A SIMPLIFIED DIAGNOSTIC TEST FOR GALACTOSEMIA

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A laboratory test is presented for the specific diagnosis of galactosemia, either at birth or later. The test requires 0.2 ml. of blood and can be completed in a few hours. It is based on the inherited deficiency of uridyl transferase and the consequent inability of hemolyzed erythrocytes to respire on galactose phosphate as subtrate.

A SURVEY being conducted at this time indicates that galactosemia is no longer a clinical curiosity: in the last few years at least 50 cases have been diagnosed in the United Kingdom (some of them post mortem). The desirability of an early diagnosis has been referred to previously.[1] All siblings of known galactosemic patients should be tested at birth. The total number of individuals who qualify for a diagnostic test may, therefore, be much greater than the number of cases reported in the literature.

The laboratory tests for galactosemia described in the literature[1, 2] are time consuming and somewhat tedious and require at least 2 ml. of blood. It was felt that a simple test requiring little blood and capable of affording a positive diagnosis in a few hours would prove useful to routine laboratories and might lend itself to screening of mental defective individuals and others.

The test described requires 0.2 ml. of blood and has been designed to use a minimum of expensive reagents. It is based on the fact, discovered by Kalekar and co-workers,[3] that normal hemolyzates, suitably fortified, respire actively on galactose-1-phosphate as substrate, whereas galactosemic hemolyzates do not. Kirkman and Bynum[4] have recently described a manometric assay of uridyl transferase based on the same principle. In our laboratory a similar assay has been developed and will be reported in due course. The experience gained with this assay has stimulated development of a simplified semiquantitative micro-version for diagnostic purposes.

MATERIAL

Principle.—The oxygen uptake of a hemolyzate is measured on galactose-1-phosphate as substrate. Controls are put up simultaneously, using (1) no substrate, (2) glucose-1-phosphate as substrate, and (3) a known normal hemolyzate plus galactose-1-phosphate. This combination of checks verifies that (1) the patient's hemolyzate does not respire without substrate; (2) the patient's hemolyzate respires normally on glucose-1-phosphate; and (3) the galactose phosphate solution is an adequate substrate for normal hemolyzates.

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Apparatus.—The apparatus consists of a thick-walled Pyrex capillary tube, about 8 cm. long, internal diameter 1 mm., which has been sealed at one end and blown into a bulb (approximate diameter 6 to 7 mm.). A mark is made on the stem 3 cm. from the sealed end of the capillary. The tube is filled with a solution of silicon,* emptied, and then dried *in vacuo*. Four tubes are required for each assay.

Reagents.—

1. Glucose-1-phosphate, dipotassium salt†: 50 mg. per milliliter.

2. Galactose-1-phosphate, barium salt†: to 3 mg. dissolved in 25 μl H_2O in a small conical centrifuge tube, 25 μl of saturated K_2SO_4 solution is added; the mixture is stirred well and then centrifuged for 10 minutes. The supernatant is transferred to a small tube or bottle which contains 2.5 mg. uridine diphosphoglucose (UDPG "90").†

3. Hemolyzate diluent: 300 mg. nicotinamide and 5 mg. methylene blue are dissolved in a mixture of 9.5 ml. 0.1M potassium phosphate buffer, pH 7.4, and 0.5 ml. 0.1M $MgSO_4$. Triphospopyridine nucleotide† (0.75 mg.) is dissolved in 0.5 ml. of the above mixture to give the diluent solution.

These three solutions can be frozen and kept for several days.

4. Approximately 5N potassium hydroxide.

METHOD

The patient's blood and that of a normal individual (0.2 ml.) are transferred to graduated conical centrifuge tubes which contain 10 to 12 ml. ice-cold saline. After this is mixed and centrifuged for 5 minutes, the clear supernatant is removed and the cells are resuspended in another 10 ml. cold saline and centrifuged 10 minutes. The supernatant is removed and the cells are frozen (they can be kept frozen for several days). After thawing, the volume of hemolyzate is estimated, or measured in a pipette, and an equal volume of diluent solution is added.

By means of a drawn-out Pasteur pipette, calibrated approximately at 3.5 and 25 μl, 3.5 μl of water, glucose-1-phosphate, and galactose-1-phospate solutions, respectively, are transferred to the bulbs of 3 reaction tubes, followed by 25 μl of diluted (patient's) hemolyzate. Another 3.5 μl of galactose phosphate solution is placed in a fourth tube and 25 μl of diluted normal hemolyzate is added. With a little practice this operation can be carried out without allowing any of the solutions to rise into the capillary stem. Should this occur, brief centrifugation will return the liquid to the bulbs.

The four tubes are placed in a water bath at 37° C. for 5 minutes with about 1 cm. of stem protruding above the water level. The tubes are then sealed by introducing enough 5N potassium hydroxide to form a liquid column 1 to 3 mm. long, with the lower meniscus close to the mark. The tubes are returned to the water bath and after a further 10 minutes' equilibration the distance of the lower of the potassium hydroxide meniscus from the mark is measured with a ruler. The zero of the ruler should be placed against the meniscus immediately after withdrawal of the tube from the bath, to ensure that the measurement is not affected by the shrinkage of the gas volume consequent upon the lowered temperature. The tubes are then incubated for a few hours, or overnight, and the position of the meniscus is then recorded once more.

INTERPRETATION

A normal hemolyzate respires both on glucose phosphate and on galactose phosphate, whereas a galactosemic one respires on the former only. The volume, as indicated by the meniscus of the potassium hydroxide in the tube of galactose phosphate, will therefore remain unchanged (±0.5 mm). The meniscus in the substrate-less (water) tube should also remain steady (or rise slightly), whereas that in the tube of glucose phosphate should drop

*Repelcote, Hopkin & Williams Ltd.
†Sigma Chemical Company.

several millimeters. The meniscus of the potassium hydroxide in the tube of galactose phosphate which contains normal hemolyzate should fall several millimeters, indicating that the substrate is satisfactory.

RESULTS

In an apparatus of the given dimensions, the respiration of normal hemolyzates gives a rate of fall of the meniscus of the potassium hydroxide of approximately 1 mm. per hour (range 0.6 to 2.3). Since the respiration ceases after a few hours, incubation overnight gives values ranging from −6 to −16 mm.

The results obtained with 7 galactosemic patients are shown in Table I.

TABLE I. GALACTOSEMIC PATIENTS (CHANGE OF POSITION OF MENISCUS IN MILLIMETERS)

PATIENT	SUBSTRATE		
	GAL-1-P	G-1-P	WATER
D. R. J.	0	− 5.0	+ 0.5
S. B.	0	− 13.5	− 0.5
C. S.	+ 0.5	− 9	0
S. J. D.	+ 0.5	− 12.5	+ 1.5
A. D.	− 0.5	− 11.5	+ 1.5
J. R.	0	− 7.5	+ 0.5
C. O. 'C.	+ 0.5	− 8	+ 0.5
Normal subjects (20)	− 6 to − 16	− 6 to − 16	− 0.5 to + 1.5

DISCUSSION

If the checks are satisfactory, an unchanged potassium hydroxide level (±0.5 mm.) establishes the diagnosis of galactosemia. On the other hand, a fall of more than 1 to 2 mm. indicates that the patient is certainly not homozygous for galactosemia. Therefore, the test furnishes the clinician with a reliable diagnosis within a few hours of birth.

The technique is such that none of the volumes or concentrations of reagents are critical, so that neither the weighings nor the pipetting need to be carried out with any high degree of accuracy. For this reason and to conserve reagents, the smallest possible volumes of expensive reagents have been used.

I would like to thank Drs. E. M. Darmady, A. Holzel, H. Everley Jones, G. M. Komrower, J. K. Magner, and J. H. Moseley for securing specimens of blood from their patients.

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FURTHER MODIFICATION OF THE BENZIDINE METHOD FOR MEASUREMENT OF HEMOGLOBIN IN PLASMA

Definition of a New Range of Normal Values*

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A method is described which minimizes artifactual hemolysis during the collection of a blood sample. Plasmas from samples so collected from normal subjects contain such low concentrations of hemoglobin as to be unmeasurable by the most sensitive available methods. Therefore, efforts were directed towards further modification of the benzidine method so as to achieve a greater than tenfold increase in its sensitivity. The resulting modified benzidine method is described in detail, with special emphasis on the relative stability of the various reagents and on the principal variables requiring strict control. The accuracy of the method for measurement of concentrations of hemoglobin in the range of 0.16 to 0.58 mg. per 100 ml. is illustrated, along with the results of recovery experiments which indicate lack of inhibitory activity in any of the normal human plasmas examined. Repeated measurements of plasma hemoglobin concentration in a resting normal subject reveal essentially no fluctuations over a 2 hour period of observation. Measurement of plasma hemoglobin concentration in 15 ambulatory and 10 sleeping normal subjects yield a mean value of 0.31 mg. per 100 ml. (range, 0.16 to 0.58 mg. per 100 ml.). The potential of the method for future studies and its importance for an understanding of normal erythrokinetics are discussed.

KNOWLEDGE of the normal plasma hemoglobin concentration is fundamental to an understanding of normal erythrokineties and of pathologic hemolytic states. There has, therefore, been a sustained interest in improving methods for quantitative measurement of hemoglobin in human plasma. Flink and Watson's pyridine hemochromogen method,[1] proposed in 1942 as a substantial advance over the Bing and Baker modified benzidine method,[2] was useful for hemoglobin concentrations above 15 mg. per 100 ml. but was subject to wide error at lower hemoglobin concentrations. In 1956, Crosby and Furth[3] described a further modification of the benzidine method which permitted accurate measurement of hemoglobin concentrations in the range of 2 to 20 mg. per 100 ml.

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Definition of the concentration of hemoglobin in the circulating plasma of normal human subjects has been impeded by two major difficulties: (1) variable artifactual hemolysis incident to collection and processing of the blood sample, and (2) lack of a method for accurate measurement of hemoglobin at concentrations below 2 mg. per 100 ml. Hartmann and Auditore[1] have recently reported that when meticulous care is exercised in the collection of blood, plasma hemoglobin values for normal subjects will generally be < 0.5 mg. per 100 ml. When we devised a method for blood collection which minimized artifactual hemolysis, the normal plasmas so obtained contained too little hemoglobin for accurate measurement by any currently available method.

The present report describes the new method for blood collection and a further modification of the benzidine method for hemoglobin measurement which increases its sensitivity tenfold. A new range of maximum normal values for plasma hemoglobin concentration is defined.

MATERIALS AND METHODS

Normal Subjects.—All subjects were either healthy medical students or laboratory personnel between the ages of 20 to 40 years.

Blood Collection.—A 12 inch length of polyvinyl tubing (3 mm. internal diameter) was attached to the hub of a sterile 15 gauge blood donor collection needle. The needle was inserted cleanly into the vein and the first 4 ml. of blood to flow freely from the tubing was discarded. Eight milliliters of freely flowing blood was then collected in each of 2 silicone-coated 12 ml. centrifuge tubes whose interiors were coated with a fine mist of heparin (1,000 units per milliliter) delivered from a simple plastic atomizer. The volume of heparin solution in each tube did not exceed 0.05 ml. No mixing of the blood with the anticoagulant solution was required. Care was taken that no blood touched the wall of the tube above the final upper level of the collected blood. The tube was promptly capped with Parafilm "M."[*]

Separation of Plasma.—Acid washed glassware rinsed 8 times in distilled water was used in all subsequent steps. The blood samples were centrifuged for 8 minutes at 2,000 r.p.m. in a table model centrifuge with a radius of 15 cm. from the center to the bottom of the centrifuge cup. The supernatant plasma was transferred to a clean glass tube and recentrifuged at 2,000 r.p.m. for 8 minutes. The plasma was transferred to a second clean glass tube; either the hemoglobin content was measured within 4 hours or the plasma was frozen and stored at −20° C. until the determination was made. Any fibrin encountered in thawed samples could be removed by centrifugation without altering the results of plasma hemoglobin measurement.

Crosby and Furth Method for Measurement of Plasma Hemoglobin.—The procedure was carried out as detailed by the authors.[3] To 1 ml. of 1 per cent benzidine base dissolved in 50 per cent acetic acid was added 0.02 ml. of solution to be tested, followed by 1 ml. of 1 per cent hydrogen peroxide solution. After vigorous mixing, the solution was allowed to stand at room temperature for 20 minutes, and 10 ml. of 10 per cent acetic acid diluent was added. The final reaction mixture was incubated for 10 minutes at room temperature and its optical density was then determined in a Coleman Junior spectrophotometer set at 515 λ.

Modified Method for Measurement of Plasma Hemoglobin.—

Preparation of purified benzidine: The purification procedure is based on the principle outlined by Bing[5]: (1) Thirty grams of benzidine base† is dissolved in 200 ml. of 70 per cent ethyl alcohol with the aid of gentle heat and allowed to recrystallize for 24 hours in the refrigerator (approximately 4° C.). The suspension of crystals is filtered with suction

[*] Marathon, a Division of American Can Company, Menasha, Wis.
† Matheson Coleman and Bell, Div. of Matheson Company, Inc., East Rutherford, N. J.

and the crystals are washed with 100 ml. of ice-cold 50 per cent ethyl alcohol. (2) The crystals are redissolved in 200 ml. of 70 per cent ethyl alcohol with gentle heat, allowed to recrystallize for 24 hours, are refiltered, and washed as previously. (3) The crystals are redissolved in 150 ml. of 70 per cent ethyl alcohol with gentle heat and after the addition of 3 Gm. of norite decolorizing carbon the mixture is heated below boiling for 5 minutes. The hot solution is filtered with suction through a heated filter, and the filter is further rinsed with 100 ml. of hot 50 per cent ethyl alcohol. To the hot filtrate is added 2 Gm. of norite; the solution is mixed and heated for an additional 5 minutes and refiltered through a heated filter with a final rinse of 100 ml. of hot 50 per cent ethyl alcohol. Crystals are permitted to form in the final filtrate during 36 hours at 4° C. (4) The crystals are then separated by a filtration with suction, washed with 100 ml. of ice-cold 50 per cent ethyl alcohol, and allowed to dry partially with suction for 30 to 60 minutes. (5) The crystals, which appear as spicules and plates of white-ivory colored material, are placed in a brown bottle and dried in a vacuum desiccator overnight. Thirty grams of benzidine base yield approximately 10 Gm. of purified benzidine. The purified benzidine may be stored for up to one month in a dry closed brown bottle; if stored for longer periods, the bottle is kept in an evacuated desiccator.

Preparation of hemoglobin standard solutions: A *stock* solution of hemoglobin is prepared by washing fresh red cells 3 times and making up to a hematocrit which will provide a hemoglobin concentration of approximately 10 Gm. per 100 ml. The suspension is hemolyzed by repeated freezing and thawing and the stroma are separated by hard centrifugation. An accurate hemoglobin determination by the cyanmethemoglobin method is then made on the supranatant hemoglobin solution and the volume of the solution is adjusted to provide a concentration of 10 Gm. per 100 ml. The stock solution is frozen and stored at −20° C. when not in use and remains stable for at least 6 to 8 weeks after preparation. With a calibrated pipette 20 c. mm. of the stock hemoglobin solution is added to 100 ml. of 0.1M sodium chloride solution in a chemically clean volumetric flask, thus providing a solution of 2 mg. of hemoglobin per 100 ml. Additional dilutions are made to provide hemoglobin concentrations of 1.5, 1.0, and 0.5 mg. per 100 ml. Since the dilute hemoglobin standards are somewhat unstable (see Results), they should be measured within 15 minutes of preparation.

Measurement of hemoglobin reagent: Three per cent hydrogen peroxide in water redistilled in glass is freshly prepared from a stock 30 per cent solution. Seven tenths per cent purified benzidine solution is made up in glacial acetic acid. When suitably purified benzidine base is employed, the solution is watery clear. The benzidine solution should be used for measurement of the hemoglobin *standard* solutions immediately after its preparation (i.e., within 10 minutes; see Results for explanation). Although slight darkening occurs on standing, the benzidine solution is suitable for measurement of *plasma* samples at any time during the 6 hours after its preparation.

Procedure: Four tenths (0.4) milliliter of plasma or other solution on which hemoglobin measurement is to be made is well mixed with 6 ml. benzidine reagent. Two tenths (0.2) milliliters of 3 per cent hydrogen solution is added, a stopwatch is started immediately, and the reagents are mixed vigorously. Exactly 3½ minutes after addition of the hydrogen peroxide reagent the optical density is read in a Coleman Junior spectrophotometer set at 700 λ. Zero optical density is determined against distilled water. A reagent blank, employing 0.4 ml. of hemoglobin-free 0.1M saline, is run at the beginning and at 20 minute intervals during a series of measurements. The optical density of the reagent blank at 3½ minutes is subtracted from the 3½ minute readings of the test samples.

RESULTS

Crosby and Furth Modified Benzidine Method.—Measurement of standard hemoglobin solutions over the range 1 to 20 mg. per 100 ml. gave results (Fig.

1) entirely comparable to those reported by Crosby and Furth.[3] It is evident from Fig. 1 that hemoglobin concentrations from 0.2 to 2.0 mg. per 100 ml. fall in the optical density range of 0.003 to 0.032, too low for accurate measurements of differences among samples in that hemoglobin range. Although some increase in sensitivity was attainable by reducing the volume of diluent and increasing the concentrations of benzidine and hydrogen peroxide as suggested by the original authors, it was not possible by these means to increase the sensitivity of the method more than threefold. Furthermore, greater instability of the reagent blanks was encountered when these modifications were employed.

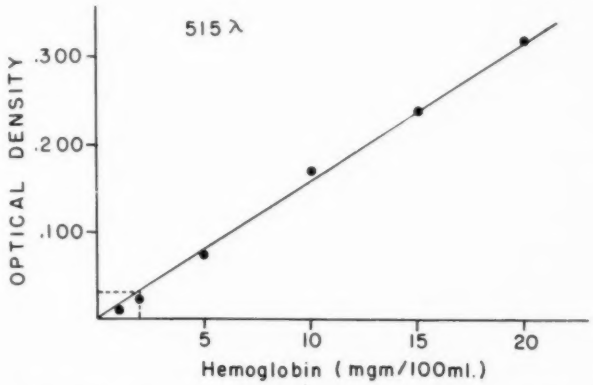

Fig. 1.—Standard curve, Crosby and Furth modified method.[3] Note that hemoglobin concentrations in the range of 0.2 to 2.0 mg. per 100 ml. fall in the optical density range 0.003 to 0.032.

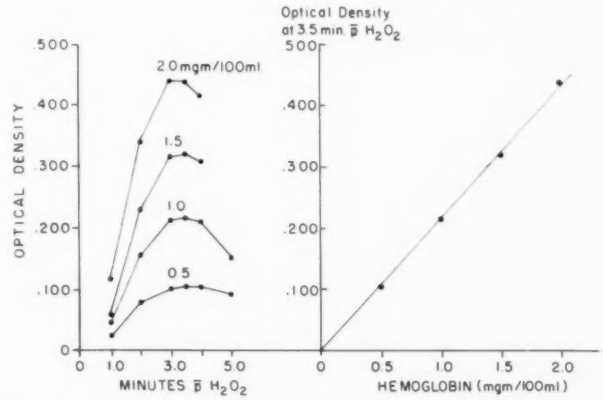

Fig. 2.—Standard curves at 700 λ, modified method. *A*, Green color peaks occur consistently at 3½ minutes after addition of hydrogen peroxide. *B*, Hemoglobin concentration of 1.0 mg. per 100 ml. yields an optical density of 0.220, compared to that of 0.016 in Fig. 1.

Development of Further Modification of Benzidine Method.—A limiting factor to increasing the sensitivity of the Crosby and Furth modification of the benzidine method was the development of turbidity in the reaction mixture if the volume of test plasma was raised above 0.04 ml. It became evident that much larger volumes of plasma could be employed if the volume of water present

in the final reaction mixture was reduced as much as possible. Thus, when the benzidine was dissolved in glacial acetic acid (rather than 90 per cent acetic acid), when the volume of hydrogen peroxide reagent was reduced to 0.2 ml. (rather than 1 ml.), and when the 10 per cent acetic acid diluent was omitted, 0.4 ml. of plasma or standard hemoglobin solution could be added without development of turbidity. When the color reaction was allowed to proceed to the final violet pigment, measured at 515 λ, considerable variation in results was encountered, and consistent linear standard curves over the hemoglobin range of 0.25 to 2.0 mg. per 100 ml. could not be obtained. Furthermore, samples of plasma evidenced at least two rates of color development, a relatively rapid initial rate followed by a prolonged slower rate, and it was difficult to know the optimum time to read the optical density.

On the other hand, when the initial green color development was measured at 700 λ for standard hemoglobin solutions, the curves shown on the left in Fig. 2 were consistently obtained. It is notable that in the hemoglobin range of 0.5 to 2.0 mg. per 100 ml. the peaks of green color development occur consistently 3½ minutes after addition of the hydrogen peroxide reagent. The peaks are sufficiently flat that little error will be introduced by readings taken at 3½ minutes ± 15 seconds. When the 3½ minute peaks are plotted against hemoglobin concentration a straight line is obtained which passes through zero. Standard hemoglobin solutions which contain 0.25 mg. per 100 ml. (not shown in Fig. 2) are still within the linear range of reaction. With respect to green color development, plasma samples uniformly showed optical density curves entirely comparable to standard hemoglobin solutions, consistently peaking at 3½ minutes. It should be noted that a solution which contains 1 mg. of hemoglobin per 100 ml. provides an optical density of 0.220 compared to 0.016 by the earlier benzidine method (cf. Fig. 1).

Stability of Reagents, Standard Solutions, and Plasma.—Hydrogen peroxide solutions remained stable for at least 6 hours after dilution with the 30 per cent stock solution. Seven tenths per cent benzidine solution was observed to give relatively high "blanks" (O.D., 0.100 to 0.110) and up to 30 per cent higher optical density values (corrected for the blank) for the hemoglobin standard solutions when used immediately after its preparation as compared with 90 minutes later. Between 90 minutes and 6 hours after its preparation, no further instability of the benzidine solution was observed. By contrast, the optical density values (corrected for the blank) for hemoglobin in *plasma* samples showed no variation, regardless of whether the benzidine solution was used immediately after its preparation or at any time during the ensuing 6 hours. Hemoglobin standard solutions which contained more than 1.5 mg. of hemoglobin per 100 ml. appeared stable for at least 6 hours after preparation. By contrast, more dilute hemoglobin standards reacted progressively less strongly with the benzidine reagent the longer the interval after their preparation from the stock hemoglobin solution. The loss of reactivity was greatest during the first hour after preparation, was greater the lower the hemoglobin concentration of the

solution, and was uninfluenced by buffering the 0.1M sodium chloride diluent over the pH range of 5.0 to 8.0. For example, measurement of a 0.4 mg. per 100 ml. standard solution would give an optical density of 0.090 immediately after its preparation and 0.060 when measured 4 hours later. Plasma samples, regardless of their hemoglobin concentration (in the range of 0.25 to 2.0 mg. per 100 ml.) showed no evidence of declining reactivity with the benzidine reagent, whether measured immediately after separation from the red cells (within 20 minutes of blood collection), after 6 hours standing at room temperature, or after several weeks stored at −20° C.

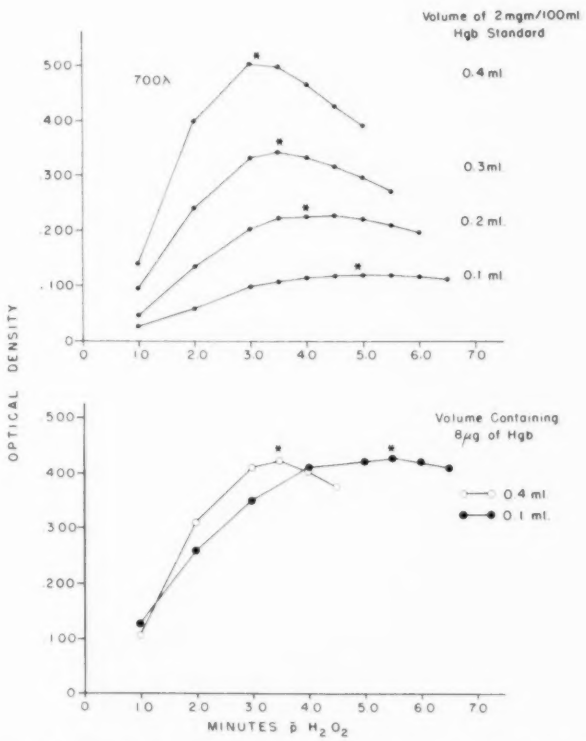

Fig. 3.—Effect of volume of hemoglobin reagent on time of color reaction peak. As the volume of hemoglobin containing solution added to the reaction mixture is decreased, the peak of green color development shifts progressively to the right. Comparison of the upper and lower graphs indicates a comparable shift to the right, independent of the absolute amount of hemoglobin added to the reaction mixture.

Factors Determining the Time of the Peak of Green Color Development.—An important factor in the determination of the rate of color development is the volume of test solution added to the reaction mixture, as illustrated in Fig. 3. It is evident from the curves in the upper graph that if decreasing volumes of test solution are employed, the peaks of green color development shift progressively to the right, in contrast to the consistent 3½ minute peak pattern shown in Fig. 2. The same phenomenon is illustrated by the curves in the lower

graph where volumes of 0.1 and 0.4 ml. of test solution were employed to deliver the same absolute amount of hemoglobin to the reaction mixture. Plasma samples which contain a high concentration of hemoglobin must be diluted sufficiently to permit the use of a full 0.4 ml. in the reaction mixture if the 3½ minute green color measurement is to be standard procedure.

Another factor which determines the time of the peak development of green color is the concentration of hydrogen peroxide employed. When a 2 mg. per 100 ml. hemoglobin standard solution was measured employing various hydrogen peroxide concentrations in the range 1.0 to 7.5 per cent, the green color peak was observed to shift progressively to the left with increasing peroxide concentrations. Optical density curves for three representative peroxide concentrations are illustrated in Fig. 4; in addition to the left shift, the sharpness of the peaks and the increasing absolute optical density values with increasing peroxide concentration should be noted. Entirely comparable shifts were observed when measurements were performed on plasma samples.

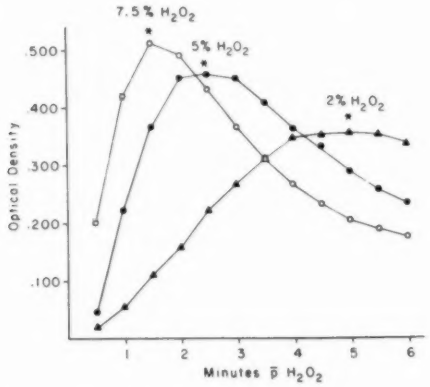

Fig. 4.—Effect of strength of hydrogen peroxide reagent on time and height of green color reaction peak for a 2 mg. per 100 ml. hemoglobin standard solution measured at 700 mμ. Note left shift of peak and increasing absolute height of peak with increasing hydrogen peroxide concentration.

When hemoglobin standard solutions are prepared in distilled water, rather than in 0.1M sodium chloride solution, the peak of green color development is shifted to the right and occurs at approximately 6 minutes after the addition of 3 per cent hydrogen peroxide solution. Similar results are obtained for plasma and urine samples which have been dialyzed overnight against distilled water. Hemoglobin standard solutions which contained 0.85, 0.6, 0.4, and 0.2 per cent concentrations of sodium chloride all showed peaks of green color development 3½ minutes after the addition of 3 per cent hydrogen peroxide. It is therefore evident that the delayed peak of color development will be observed only at markedly reduced salt concentrations (i.e., less than 0.2 per cent sodium chloride). It follows that 6 minute optical density values for test samples which have been dialyzed against distilled water should be compared with 6 minute

optical density values obtained for standard hemoglobin solutions prepared in distilled water.

Relation of Peak Height to Concentration of Chloride Ion.—When standard hemoglobin solutions are made up in increasing concentrations of sodium chloride, the absolute value for the height of the green color peak declines as the sodium chloride concentration increases. The magnitude of this effect is great, amounting to a 40 per cent fall in optical density over the sodium chloride range of 0.2 to 0.85 Gm. per 100 ml. The suppressant effect can be shown to depend entirely on the concentration of chloride ion and to be unrelated to the sodium ion concentration over the range of 0.0 to 0.15M. A similar suppressant effect is not observed for bicarbonate ion, the other major anionic moiety in human plasma. Therefore, in the preparation of hemoglobin standards 0.1M sodium chloride has been used as the diluent since its chloride concentration is comparable to that of normal human plasma.

Accuracy of the Method.—Ten measurements of plasma hemoglobin concentration were made on each of two samples of normal plasma. For the sample of lower hemoglobin content, the mean value was 0.16 mg. of hemoglobin per 100 ml. with a standard deviation of 7.7 per cent; for the sample of higher hemoglobin content, the mean value was 0.55 mg. per 100 ml., with a standard deviation of 3 per cent. Somewhat greater variation was obtained when single measurements were made on 10 separate samples of plasma obtained from a single blood collection: mean, 0.16 mg. per 100 ml. (S.D., 10.6 per cent) and 0.58 mg. per 100 ml. (S.D., 12 per cent). The latter data almost certainly reflect variations in artifactual hemolysis incident to the collection of blood in 10 separate tubes and the 10 plasma separation procedures.

Storage of Blood Sample Prior to Separation of Plasma.—To define the importance of time and temperature of storage during the interval between blood collection and the initial centrifugation, blood from a single donor was collected in 10 tubes, one of which was centrifuged immediately; the plasma separation was performed as rapidly as possible. Three groups of 3 tubes were stored, respectively, at 1 to 2° C. (ice water), at 23° C. (room temperature), and at 37° C. (warm air incubator). One tube from each group was centrifuged and the plasma was separated after 15, 30, and 45 minutes of incubation. The results of plasma hemoglobin measurement on the 10 plasma samples (Table I) indicate no effect of the time and temperature variables on the final plasma hemoglobin values.

Suitability of Different Anticoagulants.—To a single pool of normal plasma, heparinized as described under Blood Collection and having a plasma hemoglobin concentration of 0.35 mg. per 100 ml., hemoglobin was added to raise the hemoglobin concentration to 1.35 mg. per 100 ml. To 3 ml. aliquots of the pool appropriate volumes of 4 per cent trisodium citrate, "balanced" ammonium and potassium oxalate, acid citrate dextrose (U.S.P. formula A), and 5 per cent EDTA anticoagulants were added. After 15 minutes' incubation at room temperature, duplicate measurements of plasma hemoglobin concentration were

TABLE I. EFFECT OF STORAGE OF BLOOD SAMPLE PRIOR TO SEPARATION OF PLASMA

BLOOD SAMPLE STORED AT	PLASMA HEMOGLOBIN CONCENTRATION (MG./100 ML.) AFTER STORAGE FOR			
	0 MIN.	15 MIN.	30 MIN.	45 MIN.
No storage	0.45	—	—	—
4° C.	—	0.55	0.50	0.45
23° C.	—	0.60	0.30	0.30
37° C.	—	0.45	0.50	0.50

TABLE II. RECOVERY EXPERIMENTS (ADDING STANDARD HEMOGLOBIN SOLUTIONS)

PLASMA SAMPLE	HEMOGLOBIN (MG./100 ML.)		% RECOVERY
	OBSERVED	EXPECTED	
Sample A	0.32		
Sample plus 0.3 mg. per 100 ml.	0.60	0.62	93
Sample B	0.44		
Sample plus 0.3 mg. per 100 ml.	0.70	0.74	87
Sample C	0.32		
Sample plus 0.3 mg. per 100 ml.	0.60	0.62	93
Sample D	0.43		
Sample plus 0.4 mg. per 100 ml.	0.82	0.83	97
Sample E	0.55		
Sample plus 0.4 mg. per 100 ml.	0.92	0.95	92

TABLE III. RECOVERY EXPERIMENTS (ADDING HEMOLYZED PLASMA)

PLASMA SAMPLE	HEMOGLOBIN (MG./100 ML.)		% RECOVERY
	OBSERVED	EXPECTED	
Sample F	1.10		
Sample G	36.00		
1:40 dilution of G in F	1.90	2.00	89
1:80 dilution of G in F	1.50	1.55	89

TABLE IV. PLASMA HEMOGLOBIN CONCENTRATION IN NORMAL SUBJECTS

SUBJECTS	NUMBER	PLASMA HEMOGLOBIN	
		MEAN (MG./100 ML.)	RANGE (MG./100 ML.)
Ambulatory	15	0.32	0.16-0.58
Sleeping	10	0.30	0.20-0.57
Total	25	0.31	0.16-0.58

made and the results were suitably corrected for the small dilution effect of the added anticoagulant solution. The sample which contained trisodium citrate gave the expected value for hemoglobin concentration, indicating no inhibitory effect of the anticoagulant upon the benzidine reaction. In the presence of the other three anticoagulants the plasma hemoglobin concentrations were only 80 per cent of the expected value, indicating some inhibitory effect. Heparin in the amounts used throughout the present study had no effect upon the measurement of hemoglobin in plasma.

Recovery Experiments.—To five samples of plasma of known hemoglobin concentration, sufficient hemoglobin solution was added to raise the plasma hemoglobin concentration by 0.3 mg. per 100 ml. (3 samples) and 0.4 mg. per 100

ml. (2 samples). The results shown in Table II indicate a mean recovery of 92 per cent of the added hemoglobin. To 2 additional samples, the hemoglobin was added in the form of plasma of known high hemoglobin concentration, with recoveries of 89 per cent (Table III).

Plasma Hemoglobin Concentrations in Normal Subjects.—Table IV summarizes duplicate plasma hemoglobin measurements on 15 ambulatory subjects (13 men, 2 women), indicating a mean of 0.32 mg. per 100 ml. (range, 0.16 to 0.58). Samples drawn at 7 A.M. from 10 sleeping medical students revealed a mean plasma hemoglobin concentration of 0.30 mg. per 100 ml. (range, 0.20 to 0.57).

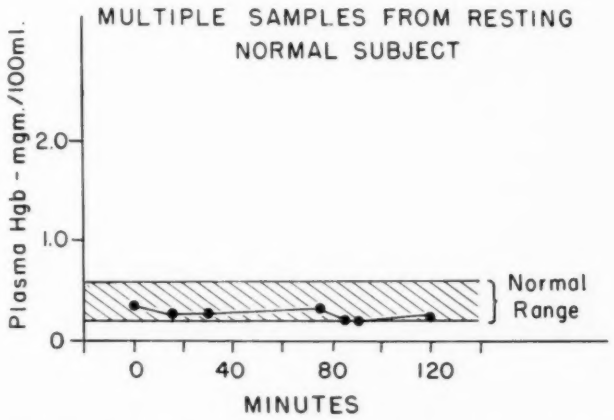

Fig. 5.—Plasma hemoglobin values varied within the range of 0.16 to 0.35 mg. per 100 ml. during the 2 hour period of observation.

To illustrate the degree of stability that might be expected in a resting normal subject, 7 blood samples were obtained at various time intervals over a 2 hour period from an indwelling 15 gauge needle kept open by a slow drip of saline which contained heparin in a concentration of 1 unit per milliliter. The results are illustrated in Fig. 5, and indicate that the plasma hemoglobin values fell within the range 0.16 to 0.35 mg. per 100 ml. during the period of observation. A somewhat wider range may be expected when multiple samples are obtained at random from an active normal subject. For example, 10 samples obtained at random intervals from one of the authors over a 10 month period yielded a mean plasma hemoglobin concentration of 0.45 mg. per 100 ml. and ranged from 0.30 to 0.58 mg. per 100 ml.

DISCUSSION

The modified benzidine method described in the present report was developed to meet the need for accurate measurement of hemoglobin concentrations in the range 0.2 to 2.0 mg. per 100 ml. It must be emphasized that the method does not appear to have any advantages over the Crosby and Furth

method[3] for measuring hemoglobin concentrations > 4 mg. per 100 ml. In fact, the latter method is to be preferred under such circumstances, since the need for dilution of the test samples is eliminated, even for samples of very high hemoglobin concentration.[6] The importance of collecting the blood samples with care to minimize artifactual hemolysis cannot be overemphasized. The present authors have no assurance that their method for blood collection has eliminated artifactual hemolysis altogether; undoubtedly it has not. However, the relatively narrow range of plasma hemoglobin values for normal subjects and the stability of plasma hemoglobin concentration in a single normal subject over a 3 hour period of observation (Fig. 4) suggest that major artifactual variations have been largely controlled.

In no instance has plasma from a normal subject, even in the presence of clearly evident alimentary hyperlipemia, caused turbidity in the benzidine reaction mixture. The plasma from one patient with essential hyperlipemia was "creamy" and caused appreciable turbidity when added to the benzidine reagent. After extraction of this plasma with 5 volumes of ether, it no longer evoked turbidity and appeared to behave normally in the benzidine reaction. Measurement of hemoglobin concentration of samples of normal plasma before and after extraction with ether showed no significant alteration as a result of the extraction procedure. The plasma from a patient with multiple myeloma (serum globulin 7.5 mg. per 100 ml.) formed a gel when it was added to the benzidine reagent and was therefore unsuitable for measurement. No studies have as yet been made to attempt to circumvent this rarely encountered difficulty.

The recovery experiments illustrated in Tables II and III emphasize the sensitivity of the method, which is capable of reliable detection of changes in hemoglobin concentration of 0.3 mg. per 100 ml. The results indicate that there was little or no free inhibitory substance in any of the 7 plasma samples included in the series. Nor has inhibitory activity been observed in any of numerous plasma samples to which known amounts of hemoglobin have been added during the course of unrelated experiments.

Preliminary studies indicate that the modified benzidine method lends itself well to measurement of heme pigments in dialyzed urine. The necessity to dialyze urine results from the known propensity of benzidine to form insoluble salts with acid radicals normally present in urine. It might be anticipated that, like urine, plasma from severely uremic patients would contain retained acids which would precipitate with the benzidine reagent. The authors have tested only two such samples from patients whose plasma nonprotein nitrogen concentrations were 210 and 156 mg. per 100 ml., respectively. A heavy precipitate was encountered when the first plasma was added to the benzidine reagent, a light precipitate developed with the second plasma. After dialysis against distilled water, the uremic plasma no longer precipitated in the benzidine reagent and was satisfactory for measurement of plasma hemoglobin concentration. Dialysis of plasma from normal subjects does not alter the value obtained

for hemoglobin concentration as long as account is taken of any dilution of the plasma that may occur during the dialysis.

It must be emphasized that the benzidine reaction is nonspecific and detects all substances which have peroxidase activity. Thus such substances as methemoglobin, myoglobin, catalase and peroxidase will all give a positive benzidine reaction. The present authors favor the interpretation that what is being measured in carefully collected normal human plasma is in fact hemoglobin. However, myoglobin may certainly be a contributing factor. It is possible that this problem can be resolved by suitable quantitative immunologic identification of one or the other of the two pigments. Studies on crystalline beef catalase indicate that a solution which contains 3 mg. of catalase per 100 ml. is required to give green color development equivalent to a solution which contains 1 mg. of hemoglobin per 100 ml. It therefore seems unlikely that physiologic concentrations of catalase in the plasma contribute significantly to the benzidine-reactive material detectable in normal human plasma.

Whether or not the benzidine-reactive material in normal plasma is entirely accounted for by hemoglobin, the new range of normal values can be considered as *maximum* values for plasma hemoglobin concentration. Minimization of artifactual hemolysis incident to blood collection is thought to be responsible for the low range of normal values in the present study—0.16 to 0.58 mg. per 100 ml. in contrast to 1.0 to 4.0 mg. per 100 ml. previously reported by Crosby and Dameshek.[7] Garby and Noyes[8] have recently presented a mathematical model which satisfies the plasma disappearance curves they obtained after induced hemoglobinemia in the range of 10 to 200 mg. per 100 ml. of plasma. Preliminary hemoglobin disappearance curves obtained by the present authors over the range 0.2 to 3.0 mg. per 100 ml. appear to fit Garby's model closely. From a knowledge of the daily hemoglobin turnover (approximately 6 to 7 Gm.) and of the normal level of plasma hemoglobin, the mathematical model permits an estimation of the per cent of the normal daily destruction of red cells which occurs intravascularly. If the normal plasma hemoglobin concentration is 0.3 mg. per 100 ml., it is estimated that less than 10 per cent of the normal destruction of red cells occurs by release of hemoglobin directly into the circulating plasma. This finding has important implications for a general understanding of erythrokinetics, especially when considered along with Bessis'[9] striking demonstration of the transfer of iron directly from phagocytized red cells to early erythroblasts through the mediation of reticulum cells in the bone marrow.

The stability of the plasma hemoglobin concentration in the resting normal subjects bodes well for subsequent studies of various physiologic stresses which might provoke a rise or a fall in the plasma hemoglobin concentration in normal subjects. Detailed studies of the effects of circulatory stasis, heat, cold, sleep, exercise, dietary intake, and other factors are in progress in our laboratory and will be the subject of a second report. When the factors which determine the plasma hemoglobin concentration in the normal subject have been more completely defined, the method should lend itself well to studies of pathologic hemolytic states.

SUMMARY

1. A method for blood collection designed to minimize artifactual hemolysis has been described. No mixing of the blood sample with its anticoagulant is required.

2. A tenfold increase in sensitivity of measurement of hemoglobin has been achieved by further modification of the benzidine method.

3. Application of the modified benzidine method to measurement of hemoglobin in human plasma has been described. A new range of maximum values for plasma hemoglobin concentration in normal subjects has been defined: 0.16 to 0.58 mg. per 100 ml., with a mean value of 0.31 mg. per 100 ml.

4. Some implications of the newly defined normal range have been briefly discussed.

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